THE JOURNAL OF GENERAL PHYSIOLOGY

Founded by Jacques Loeb

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VOLUME FORTIETH
WITH 2 PLATES AND 369 FIGURES IN THE TEXT

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1957

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THE NON-CORRELATION OF BIOELECTRIC POTENTIALS WITH IONIC GRADIENTS*

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(Received for publication, January 23, 1956)

Present day theories postulate that the resting potential (R.P.) is essentially determined by the ionic gradients between the interior of the cell and its exterior. This asymmetry is said to be produced at least partly by a Donnan effect (1) and partly by a specific metabolically mediated Na^+ extrusion (2). Thus, Hodgkin and Katz (3) were able to account for the dependence of the resting potential in the squid giant axon on the external K^+ by adapting the constant field equation of Goldman (4). Data on resting potentials of muscle fibers (5, 6) are in agreement with this analysis.

Alteration of the external ionic environment was also a principal tool in the explanation provided by Hodgkin and Katz (3) for the overshoot of the axonal spike. According to these authors this response is initiated by a small decrease of the resting potential which causes in turn a striking, specific alteration in the permeability of the membrane first to Na^+ and then to K^+ ions. The suggestion has been made that the rising phase of the action potential is due to the penetration of a small amount of Na^+ ions, discharging the membrane to a value which is generally close to the sodium diffusion potential. Goldman (4) predicted that the effect of K^+ and Cl^- permeability would be to reduce the spike height when the Na^+ gradient is steep.

The theory that the bioelectric potentials are directly related to the ionic ratios across the membrane has resulted almost entirely from a consideration of the results of the alteration of the external environment. Only recently have workers turned their attention to the effect of change of the internal ionic composition. Desmedt (7) has studied the effect of increased internal Na^+ on the potentials, and his results are in agreement with the classical theory. However, a close examination of his paper reveals inconsistencies, which will be discussed later.

Grundfest *et al.* (8) have devised a microinjection technique to bring about alteration of the internal ionic constitution, and they have found that alterations of the internal K^+ or Cl^- concentrations do not cause the change in resting potential expected on the basis of a Donnan mechanism.

* This work was carried out with the aid of a grant from the National Health and Medical Research Council of Australia.

The work to be described in this paper takes advantage of the naturally occurring variation in the internal Na^+ and K^+ level of the sartorius muscle of the toad, *Bufo marinus*. In an earlier communication (9), it was shown that there is a temporal variation of several hundred per cent in the Na^+ content. There is a small, but by no means concomitant K^+ variation. In this paper these ionic variations will be correlated with the electrical potentials.

Methods

Material.—The experiments reported here were made throughout 1955 on the toad *Bufo marinus*, batches of which were obtained at fortnightly intervals. The brain and spinal cord of the animals were destroyed. The skin over the sartorius muscle was slit and pinned back to form a bath. In the earliest experiments the "bath" was filled with paraffin oil, but later a small amount of Ringer was placed in the bath (if there was insufficient extracellular fluid to cover the muscle). Isolated muscles were prepared by careful dissection.

Solutions.—Either toad Ringer or phosphate Ringer was used. The composition of these solutions is given in an earlier paper (9) with the exception that the total phosphate was reduced to 3 mM/liter in the phosphate Ringer, while the Na^+ was kept at 130 mM/liter in both solutions.

Soaking Procedure.—Isolated muscles were soaked in 30 ml. solution until they had achieved a steady state with respect to Na^+ and K^+, a process taking from 2 to 3 hours (9).

Apparatus and Electrical Recording.—Penetrations were done under microscope control with Ling-Gerard type microtips, filled with 3 M KCl.

Recording equipment consisted of a D.C. amplifier with a high impedance low capacity cathode follower (1000 meg. and 1 $\mu\mu$ fd.) input. The rise time of the apparatus was frequently checked with a square wave derived from the stimulator and injected in series with the microtip. The over-all rise time lay between 75 and 125 μsec.

Electrolyte Estimation.—See Shaw and Simon (9).

RESULTS

Individual Variation in Ionic Content.—We have analyzed 320 muscles during the last 4 years. The results are given in Figs. 1 and 2 and Table I. The mean value of the total Na^+ is 33.4 m.eq./kg. but 57 per cent of our muscles contain less than 29 m.eq./kg., and 7 per cent more than 46 m.eq./kg. In contrast to this wide variation the K^+ content is comparatively constant. The mean is 87.7 m.eq./kg., the lowest figure 71.0 m.eq./kg., and the highest 112 m.eq./kg. We feel confident from this fact, and other evidence (9), that the variation in the Na^+ analyses is not due to analytical inaccuracy.

A similar variation in the Na^+ and constancy of the K^+ content, has been found in the results of our analyses of nerve (9). In addition the Na^+ content of muscles of one toad tends to follow the content of the same ion in the nerve of the same animal (see Table I).

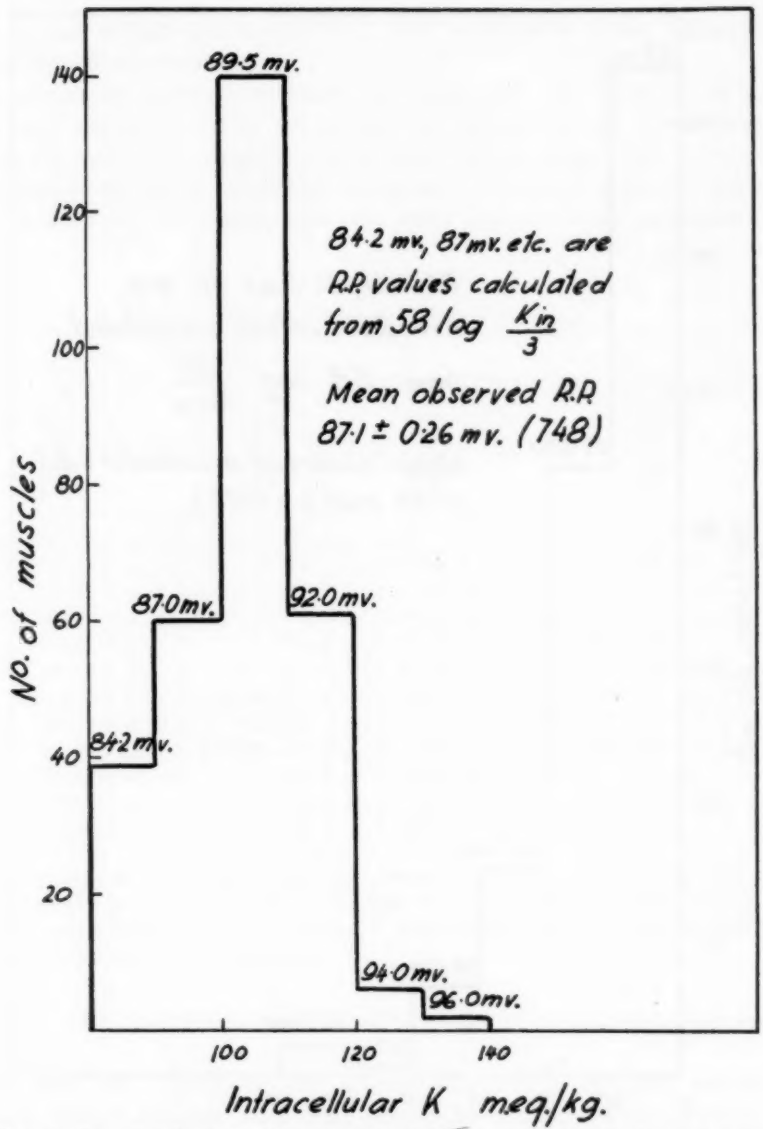

FIG. 1. The distribution of intracellular Na^+ in sartorii. This figure is compiled from analyses carried out over 4 years.

Analyses of plasma have also been made from time to time, but these show that the Na^+ content is constant at 130 ± 2.0 (16 observations) m.eq./liter and the value for K^+ is 3.0 ± 0.08 (18 observations).

The point now arises whether the intracellular Na^+ content shows a simi-

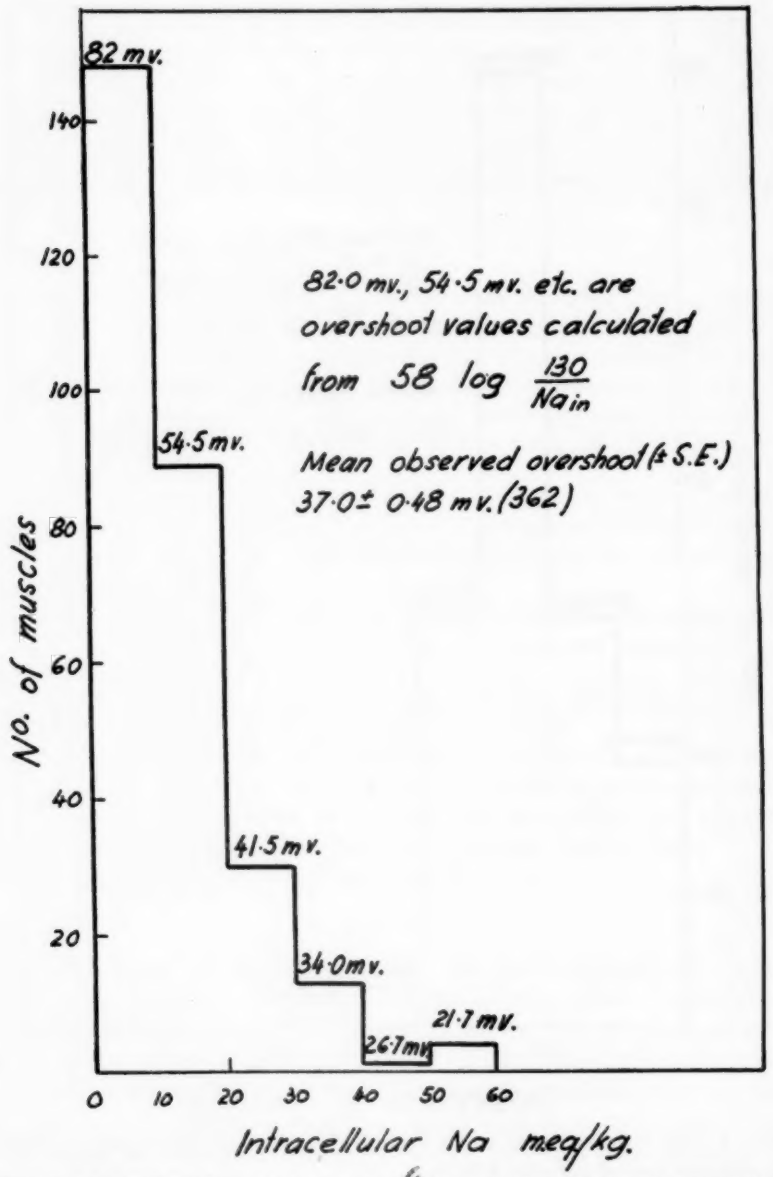

FIG. 2. The distribution of intracellular K^+ in sartorii. This figure is compiled from analyses carried out over 4 years.

lar variation to that of the total Na^+. This would be so if the extracellular volume remained constant.

Other possible inaccuracies have been considered; for instance, our analyses have not been carried out on fat-free tissue, but several estimations of the fat content of sartorius muscles gave values of less than 1 per cent. Variations in the water balance of the animal could also not offer an explanation, because (*a*) the animals were kept moist and (*b*) similar variations were

TABLE I

Temporal Variation in Ionic Content

Month	Sartorii				Sciatics			
	H_2O, *gm./kg. wet weight*	Na, *m.eq./kg. wet weight*	Intracellular Na, *m.eq./kg.*	K, *m.eq./kg. wet weight*	H_2O, *gm./kg. wet weight*	Na, *m.eq./kg. wet weight*	Intracellular Na, *m.eq./kg.*	K, *m.eq./kg. wet weight*
July	814	20.8 ± 4.4 (12)	0	70.9 ± 2.3	790	60.6 ± 2.7 (12)	0	24.4 ± 1.7
Mar.	786	20.8 ± 1.7 (8)	0	88.2 ± 2.7	775	91.5 ± 9.3 (8)	45.0	34.1 ± 4.8
Sept.	789	26.9 ± 1.2 (12)	7.3	98.0 ± 1.1	742	74.0 ± 2.1 (12)	10.0	37.1 ± 1.0
Nov.	813	27.4 ± 1.7 (8)	7.9	90.0 ± 1.4	773	71.8 ± 1.3 (8)	5.6	30.7 ± 0.8
Oct.	840	29.0 ± 1.8	9.8	78.3 ± 4.3	798	73.4 ± 4.4 (4)	8.8	37.8 ± 1.7
Jan.	806	30.6 ± 1.2	11.6	85.4 ± 2.6	772	68.5 ± 3.2 (8)	0	22.8 ± 3.8
Oct.	816	32.9 ± 1.2 (10)	14.3	74.6 ± 1.4	797	68.6 ± 2.0 (10)	0	25.3 ± 1.4
Aug.	797	33.7 ± 3.9 (10)	15.3	89.4 ± 2.8	796	63.3 ± 2.0 (10)	0	36.4 ± 2.1
Apr.	806	33.9 ± 1.2 (10)	15.6	92.6 ± 2.2	799	85.6 ± 3.4 (10)	232	34.2 ± 1.5
May	825	36.9 ± 3.6 (10)	19.1	82.8 ± 4.1	818	85.2 ± 2.3	22.4	41.3 ± 1.0
Oct.	826	45.1 ± 2.7 (6)	27.6	68.8 ± 3.1	802	90.1 ± 1.1 (4)	42.2	40.4 ± 2.5
Dec.	816	54.3 ± 2.6 (8)	39.6	90.4 ± 2.0	821	112.4 ± 6.6 (8)	86.8	34.7 ± 0.9

The intracellular Na in sartorii has been calculated on the basis of 15 per cent extracellular volume and in the sciatics on the basis of 50 per cent extracellular volume. The figures in parentheses refer to the number of observations. This table was compiled from experiments carried out over 15 months.

obtained when the results of the analyses were calculated on a dry weight basis.

We have not carried out any determinations of extracellular volume of muscle. This is because there is at present no method for determining this value with any degree of satisfaction. Most of the results in the literature suggest that it is around 15 per cent (7, 14). Errors in estimation of the extracellular volume will produce greater variation in the calculated intracellular Na^+ than in the calculated intracellular K^+ due to the very much greater Na^+ content of extracellular fluid. If variation in extracellular space is to account for the differences in Na^+ content, then those mus-

cles showing a low Na^+ content, and having presumably a low extracellular space, should have a raised K^+ content, and conversely those muscles showing a high Na^+ content, and so having a high extracellular space, should have a low K^+ content. As can be seen from Table I this correlation is not found. Furthermore, it is possible to calculate the variation in extracellular space that would be necessary if there is in fact a constant intracellular level of Na^+. We shall assume this level to be approximately 29 m.eq./liter Na, a value which, when substituted in the Nernst equation gives the observed

TABLE I *a*

Individual Variation in Ionic Content of Sartorii

Total water, *gm./kg. wet weight*	Na, *m.eq./kg. wet weight*	Intracellular Na	K, *m.eq./kg. wet weight*
817	15.0	−5.9	76.8
816	16.9	−3.6	79.2
808	19.4	−0.7	72.5
799	20.5	0.6	77.2
807	18.3	−2.0	74.4
810	20.5	0.6	73.4
812	17.3	−3.2	82.2
813	20.0	0.0	78.6
818	21.9	2.2	81.4
826	26.8	8.0	76.5
815	30.6	12.5	72.5
830	22.8	3.3	80.0

These figures correspond to July group in Table I. Paired muscles were taken from toads.

result of 37 mv. for the overshoot. The range of total Na^+ content varies from 16 to 75.0 m.eq./kg. (wet weight). It is obvious for the low figure that even if there were no extracellular space there would not be sufficient Na^+ to obtain the ratio required by the equation. In muscles with the high Na^+ we must assume an extracellular space of 33 per cent to give an intracellular level of 29 m.eq./kg. Returning to a consideration of the low Na^+ (16 m.eq./kg) muscles, it is easily seen that if a constant value of 13 per cent for the extracellular volume is assumed (14), then these muscle fibers will, of course, contain a negative concentration of Na^+ (Table I *a*). This is impossible, so we are forced to assume that the extracellular volume is a variable with a minimum value of 10 per cent. At this value for the volume of the

extracellular space there would be little or no intracellular Na^+. This is demonstrated in Table I *a*, in which the intracellular Na^+ for muscles of the month of July has been calculated on the basis of 15 per cent extracellular volume, giving negative values in some individual muscles. Thus the maximum value of the extracellular space in these muscles can be calculated as that volume which would give zero intracellular Na^+. It will be seen in Tables I and I *a* that there is no correlation between variation in Na^+ and water content of the tissue, which remains constant over the whole series.

Further evidence that there is a genuine variation in the intracellular Na^+ is provided by the behavior of the muscle when soaked in Ringer. Those muscles which are low in Na^+ gain more Na^+ than those of average content, while the high Na^+ muscles extrude Na^+ into the Ringer solution. That is, all muscles tend to assume a similar Na^+ concentration when soaked in Ringer. This phenomenon has also been observed with sciatic nerves (9).

Bioelectric Potentials.—We have found a value of 87.4 ± 0.38 mv. for the resting potential (491 observations), and of 37.0 ± 0.32 mv. for the overshoot (433 observations) for isolated muscles soaked in Ringer. These values are in agreement with those obtained by other workers in this field, and are also reasonably close to the potentials predicted by the Nernst equation on the basis of the ionic gradients across the membrane found in soaked muscle.

In view of the fact that there is a larger variation in the intracellular Na^+ content *in vivo* than there is in the isolated soaked muscle, and that the isolated muscle gains Na^+ and loses K^+, we thought it advisable to compare the potentials measured in the intact animal with those obtained when the muscle is isolated. We were impressed with the constancy, first, of the resting potential in both the *in vivo* and isolated preparations, and second, with the even more remarkable similarity of the overshoot in both preparations, although in the *in vivo* preparations there were great variations in the ratio $\frac{Na^+_{out}}{Na^+_{in}}$. Over a period of a year the overshoot measured in dissected muscles bathed in Ringer solution gave a mean value of 37.0 ± 0.32 mv. (433 observations in 24 muscles). When we came to measure the potentials in the sartorius muscle *in situ* in a pithed toad (the capillary circulation could be observed under the microscope), it was found that the scatter of the overshoots was much greater than that observed with isolated preparations. The maximum potentials obtained (there were a few as high as 50 mv.) were no greater than those found in the isolated preparation, but there were many values in the region of 10 to 20 mv., values never obtained with the isolated preparation. The configuration of these action potentials was sometimes abnormal, and led us to suspect the presence of an artifact. A perusal of the paper by Easton (11) suggested to us that in the *in vivo* preparation there was interaction between the fiber in which the electrode was situated and adjacent fibers.

This hypothesis was checked by withdrawing the electrode just outside the fiber and recording a potential on restimulation. This resulted in a diphasic wave. This irregularity corresponded in time and amplitude to a similar irregularity which could be observed on the resting potential line when the fiber failed to "fire," and parts of it could be detected in the latent period or afterpotential when the fibre did fire. We therefore assumed that on some occasions this "extrafibrillary potential" would occur during the action po-

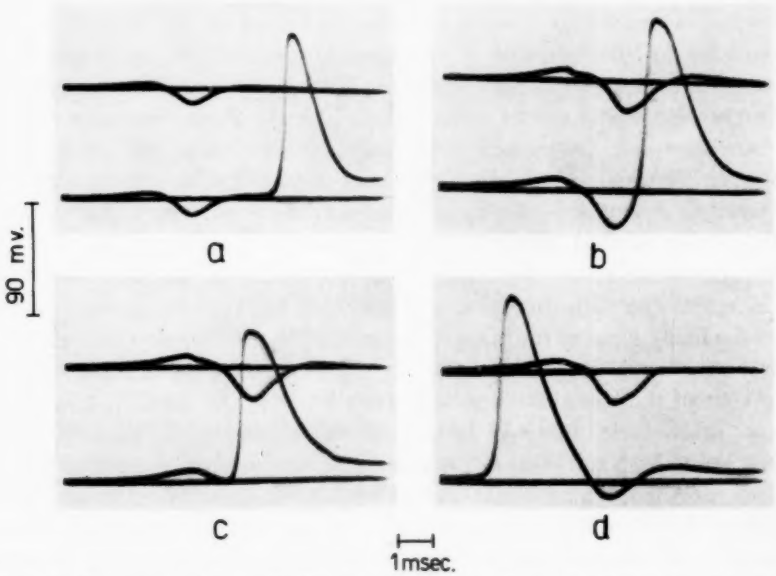

FIG. 3. Examples of extrafibrillary potentials. *a* shows resting potential, action potential, and extrafibrillary potential superimposed in one photograph. *b*, *c*, and *d* are further examples from another muscle, in which the extrafibrillary potentials occur at different time periods and lead to distortion of the action potential.

tential (A.P.) and would sum with it. Our procedure therefore was as follows: the resting potential of the cell was measured, the muscle was then stimulated directly, and the action potential recorded, the electrode was then withdrawn a slight distance from the membrane and the muscle restimulated. The four photographs were taken on the one frame, and the overshoot was measured from the peak of the action potential to the corresponding point, vertically below, on the extrafibrillary potential (see Fig. 3). The nature of the extrafibrillary potential has been investigated more throughly than has been described above, and the justification for the summation carried out here will be the subject of another paper (12). Briefly, the size of the extrafibrillary potential was not reduced if the toad were cura-

rized, but was lessened to one half and sometimes was absent altogether if, as sometimes happened, the muscle were covered with a deep layer (2 mm.) of extracellular fluid, or Ringer solution were placed in the skin flap "reservoir" to about the same depth. When these corrections for the extrafibrillary potentials were made the large scatter of the values of the overshoots was reduced; *i.e.*, the low values (10 to 20 mv.) were not obtained but high values (about 45 mv.) were found to be unaccompanied by extra-

TABLE II

Na Content and Overshoot in Isolated and in Vivo Sartorii

In vivo		Isolated	
Intracellular Na, *m.eq./kg. wet weight*	Overshoot, ±S.E. *mv.*	Intracellular Na, *m.eq./kg. wet weight*	Overshoot, ±S.E. *mv.*
10.1	29.0 ± 1.8 (21)	41.9	34.8 ± 1.2 (18)
11.3	42.0 ± 1.2 (24)	42.0	41.0 ± 0.9 (29)
14.7	38.9 ± 2.1 (17)	36.8	33.2 ± 1.6 (13)
18.5	42.8 ± 1.4 (26)	42.1	37.1 ± 1.6 (19)
18.8	41.5 ± 0.9 (67)	35.7	39.8 ± 0.9 (39)
23.8	37.7 ± 1.9 (15)	32.1	36.6 ± 1.4 (12)
24.1	35.6 ± 1.9 (24)	44.6	33.9 ± 1.4 (28)
27.7	32.8 ± 1.3 (29)	54.0	35.8 ± 0.8 (34)
29.8	35.7 ± 2.1 (18)	52.8	33.4 ± 2.0 (22)
30.4	34.9 ± 1.1 (31)	39.2	38.7 ± 1.1 (18)
32.6	37.8 ± 2.9 (20)	18.5	30.8 ± 1.2 (17)
35.2	44.0 ± 0.5 (14)	32.7	36.8 ± 1.8 (16)
35.7	33.2 ± 1.4 (17)	32.5	35.7 ± 3.3 (11)
36.2	34.9 ± 1.2 (22)	58.0	40.2 ± 1.1 (22)
48.9	37.2 ± 2.6 (12)	41.0	33.7 ± 1.3 (16)
53.4	33.5 ± 2.2 (16)	38.0	40.0 ± 0.8 (10)
57.8	33.8 ± 1.9 (19)	37.8	42.8 ± 1.1 (22)
68.0	39.0 ± 1.4 (17)	36.1	33.2 ± 1.3 (16)

fibrillary potentials. The fact that the individual variation was decreased may also be taken as a justification of our procedure.

Extrafibrillary potentials were sometimes obtained in isolated preparations, but when present were considerably smaller (approximately 4 mv.). The isolated muscles were always covered with a layer of Ringer solution several millimeters deep; if this were replaced by paraffin oil the extrafibrillary potentials were markedly increased. This is in conformity with the results obtained *in vivo*.

It should be stressed that there is no difficulty associated with the measurement of the resting potential in the *in vivo* preparations.

The results obtained for the values of the resting potential and overshoot

in vivo and in isolated preparations have been compared in Tables II and III. Figs. 4 and 5 show the plot of resting potential and overshoot against

TABLE III

K Content and Resting Potential in Isolated and in Vivo Sartorii

In vivo		Isolated	
Intracellular K content, *m.eq./kg. wet weight*	R.P., mv. S.E.	Intracellular K content, *m.eq./kg. wet weight*	R.P., mv. S.E.
83.5	88.4 ± 1.0 (18)	75.7	90.8 ± 1.1 (22)
85.6	76.8 ± 1.2 (11)	73.8	82.5 ± 1.1 (11)
91.5	87.2 ± 1.0 (26)	73.5	85.3 ± 0.8 (19)
94.3	88.5 ± 1.2 (24)	76.5	87.8 ± 1.1 (29)
96.2	90.0 ± 1.6 (19)	83.0	99.1 ± 1.3 (7)
97.0	91.0 ± 1.7 (21)	90.0	93.6 ± 1.2 (14)
98.5	87.8 ± 1.9 (6)	89.2	84.1 ± 2.2 (10)
99.3	81.2 ± 1.5 (14)	93.8	88.7 ± 1.5 (8)
101	88.8 ± 1.4 (24)	88.0	88.0 ± 1.2 (28)
101	89.1 ± 1.2 (29)	76.0	80.2 ± 0.7 (34)
101	90.7 ± 0.5 (20)	97.5	85.4 ± 0.9 (17)
101	89.6 ± 1.2 (20)	93.2	99.0 ± 2.8 (18)
102	87.0 ± 1.2 (22)	94.0	85.2 ± 1.7 (22)
103	86.6 ± 3.3 (8)	72.0	92.7 ± 1.2 (16)
105	85.3 ± 2.2 (16)	89.8	87.0 ± 0.7 (16)
109	89.2 ± 1.6 (17)	93	90.3 ± 1.1 (13)
109	86.6 ± 0.6 (68)	88.4	85.4 ± 0.8 (39)
110	90.5 ± 0.9 (14)	86.5	82.7 ± 1.0 (19)
111	88.6 ± 1.3 (12)	90	89.2 ± 1.7 (10)
112	89.0 ± 1.3 (16)	109	83.0 ± 3.9 (22)
112	90.4 ± 1.6 (15)	103	90.2 ± 1.0 (12)
113	83.0 ± 2.4 (8)	78.0	79.5 ± 1.5 (6)
114	87.2 ± 0.5 (16)	100	86.4 ± 0.4 (16)
114	91.0 ± 1.3 (19)	100	87.5 ± 1.1 (22)
115	88.7 ± 1.2 (11)	90.5	84.4 ± 1.2 (9)
117	84.0 ± 1.0 (21)	94.6	84.6 ± 1.2 (18)
122	89.7 ± 1.4 (15)	87.3	99.4 ± 5.1 (13)
124	84.0 ± 1.2 (16)	88.0	90.8 ± 0.8 (10)
125	87.3 ± 1.0 (13)	102	90.6 ± 1.0 (16)
126	86.6 ± 3.2 (8)	92.3	84.7 ± 1.3 (14)
126	88.4 ± 2.7 (16)	98.8	84.5 ± 1.9 (8)
128	84.0 ± 0.9 (19)	88.1	87.4 ± 1.2 (12)
132	88.4 ± 1.3 (31)	104	85.2 ± 1.2 (18)

log K^+_{in} and log Na^+_{in} respectively. It will be seen that while the intracellular Na^+ varies from 10.1 to 58.0 m.eq./kg., the K^+ only varies from 72.0 to 132 m.eq./kg. The overshoot does not show any variation such as is shown by the intracellular Na^+, but remains comparatively constant throughout the series of muscles examined (37 mv. ± 0.48, 362 readings).

The resting potential has also remained comparatively constant, although the intracellular K has varied from 72.0 to 132 m.eq./kg. (87.1 mv. ± 0.26, 748 readings).

The best fitting line for fig. 4 was found to be

$$y = 83.35 + 4.04 \log x$$

The standard error of the regression coefficient b is 11.92, showing that

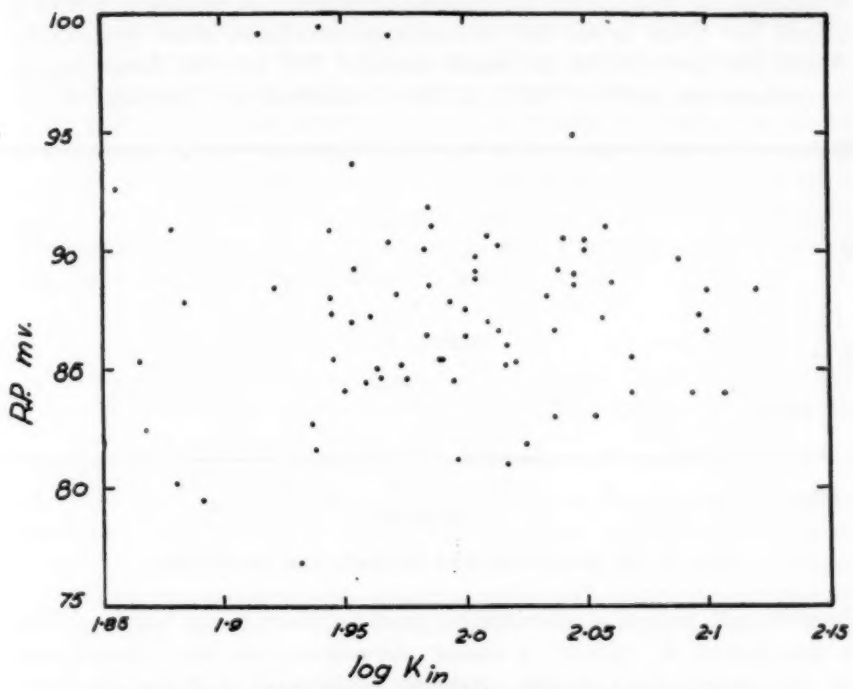

FIG. 4. The relation between log K^+_{in} and resting potential.

there is no evidence of correlation between y and log x. Moveover, the value $b = 4.04$ differs significantly from 58, the theoretical value. This shows that the lack of agreement between theory and experiment is not simply due to variability in the recorded measurements of the resting potential, assuming always that the experimental technique is adequate. The intercept $a = 83.35$ has standard error 9.26 and is undoubtedly different from the postulated value. One concludes that it is virtually impossible for a phenomenon governed by the Nernst law to throw up data such as have been obtained.

The linear regression in Fig. 5 is given by

$$y = 37.67 - 0.91 \log x,$$

with the regression coefficient $b = -0.91$ not significantly different from zero but significantly different from the postulated value -58. The standard error of b here is 5.09. The intercept $a = 37.67$ has a standard error 2.94.

In Tables II and III the potentials found *in vivo* in one muscle have been compared with the potentials obtained for the muscle taken from the other leg, which had been soaked in Ringer for a period of at least 2 hours.

The results have been listed in order of increasing intracellular Na^+ concentration (*in vivo*). When the companion muscle was soaked in Ringer it gained Na^+ if the *in vivo* Na^+ concentration was below about 50 m.eq./kg. Above this concentration the muscle extruded Na^+ into the Ringer against a concentration gradient. This is another example of the "pumping" of Na^+

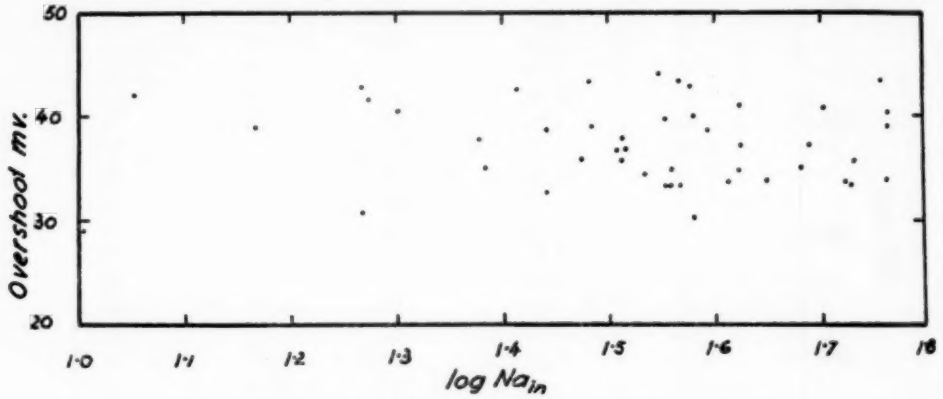

FIG. 5. The relation between log Na^+_{in} and the overshoot.

from the cell, against a concentration gradient, which is not associated with a concomitant K^+ uptake. A similar phenomenon has been demonstrated by the authors when muscles previously equilibrated in Ringer are transferred to Ringer with half the Na^+ replaced by sucrose (13).

When the muscles showed a gain of Na^+ on soaking there should, of course, have been a decrease in the value of the overshoot. Our results show that there was no significant difference in the measured potentials. Those muscles which extruded Na^+ should have shown an increase in the value of the overshoot. This could not be demonstrated.

Implicit in these remarks is the notion that gain of Na^+ means transfer to an aqueous medium inside the region across which the potential difference occurs. Obviously, available methods do not give this spatial information. From this one may take the extreme viewpoint that analytical data for total ionic content are not adequate for proving or disproving an ionic theory for the transmembrane potential. There may be binding of ions intra-

cellularly and, less likely, extracellularly. Evidence to be presented later (10) would indicate that binding of Na^+ at least, is unlikely.

All the muscles in this series showed a very significant decrease in K^+ content when soaked in Ringer. The average loss in K^+ was 17.4 m.eq./kg. per pair. This would correspond to a lowering of the resting potential by 4.5 mv. (calculated from the Nernst equation). Over the whole series there was no statistically significant difference between the resting potential of either preparation. The resting potential *in vivo* was 87.1 mv. $\pm$ 0.26 (748 observations), and the resting potential in the isolated preparation was 87.4 $\pm$ 0.38 mv. (491 observations), thus it should have been possible to detect the predicted drop in resting potential on soaking the muscle.

The rate of rise of the action potential was remarkably constant in all the *in vivo* preparations (320 volts per second). The rate of rise of the action potential in isolated preparations was, in two cases out of three, the same as that found *in vivo*. At other times it was slower (240 volts per second). The duration of the action potential was always increased in the isolated preparation (*in vivo* 2 msec.; isolated 3 msec.).

DISCUSSION

Our results clearly show, by means of two criteria, that there is no correlation such as would be expected from the Nernst equation, between the ratio $\frac{K^+_{in}}{K^+_{out}}$ and $\frac{Na^+_{out}}{Na^+_{in}}$ and the resting potential and action potential respectively. First, as will be seen from Figs. 4 and 5, a comparison of the results of the analyses for K^+ and Na^+ of individual muscles with the bioelectric potentials obtained shows an absence of correlation. Second, the alteration in ionic content of muscles which takes place on soaking is not reflected by a change in the bioelectric potentials which remain unaltered (see Tables II and III).

In no respect are our measurements at variance with those which have been obtained by other workers in the past. It will be noticed that over the small range of intracellular Na^+ and K^+ which is most frequently found in Ringer soaked muscles there is good agreement between the experimental and theoretical values for the resting potential and overshoot. It is only when the potentials are measured in cells whose ionic content varies markedly from the average that the non-correlation becomes apparent. In particular, it should be noted from Fig. 1 that the distribution of internal Na in the intact animal is such that only 17 per cent of muscles should give, from the Nernst equation the observed overshoot of 37 mv. 76 per cent of muscles should give a higher overshoot (more than 55 mv.) and 7 per cent of muscles of the series should give an overshoot less than 26 mv. It would appear that in the past other workers in this field have been content to use average values

of ionic content for their calculations of potential. These average values were obtained at different times and in different parts of the world.

It now remains to deal with the possible errors in our results. The major possible source of error in the measurement of the action potential *in vivo* is the extrafibrillary potential. We must leave a justification of our procedure to another paper (12). This error does not apply to recordings made with isolated preparations nor to measurements of the resting potential. We also know that there is an error associated with the method of recording by means of microtips, but this should not, in such a large series, obscure any definite trends in the observed potentials.

There could possibly be a variation in the ionic content of the extracellular fluid. This we have checked by making frequent analyses of the plasma, and occasional analyses of the tissue fluid whenever a sufficient quantity could be obtained. The analyses showed that the variations were not great, being usually of the order of a few per cent, and never greater than 15 per cent. This objection would not apply to isolated muscles, in which we observed a similar lack of correlation.

The potential readings were, of course, made on cells contained in the first few layers of the upper surface of the muscle, while the analysis would give the mean ionic content of all fibers. There is, however, no evidence that the ionic composition of the surface cells is different from that of cells more centrally situated. There was no deterioration of the surface fibers with time, as constant results were obtained for up to 2 hours from the commencement of the experiment.

The values calculated for the intracellular Na^+ and K^+ will depend upon the value assumed for the extracellular volume. The difficulties associated with this estimation have been discussed earlier in the paper. We have not calculated the concentrations on the basis of concentration in the fiber water because we do not consider it possible to make adequate allowance for the extracellular solids in the calculation of the fiber water. This will, of course, affect our absolute values, but it will not influence the results in which we have compared isolated and *in vivo* preparations, as each result will be altered by a similar amount.

The fact that the low theoretical values of the overshoot are not obtained experimentally when we have a high intracellular Na^+ could result from a binding of part of the Na^+, or a considerable reduction of the activity of this ion in these muscles. This explanation cannot be used to account for normal values of the overshoot which are found in muscles which contain little intracellular Na^+.

Desmedt (7) is the only other worker who has determined bioelectric potentials and ionic contents on the same preparations. The plot of Na^+_{in} against overshoot published by this author shows a lack of correlation at low Na^+_{in} levels. This he ascribes to the effect of K^+ and Cl^- permeability on the height

of the overshoot. We have also obtained results with low Na^+ muscles which do not agree with theory, but in addition we found non-correlation in high Na^+ muscles. The invoking of a permeability change is less likely to be applicable here. The reduction of the overshoot at high Na^+_{in} levels was found by Desmedt on muscles which had been held at low temperature in reduced K^+ Ringer for several days. This treatment admittedly leads to deterioration of surface fibers; and potentials obtained from deeper fibers are always subject to distortion by extrafibrillary potentials. Further, the increases in overshoot reported by Desmedt when the muscles are returned to room temperature in high K^+ Ringer, in only two of the four experiments reported, are proportionally as great as the Na^+ extrusion, and may in fact parallel the improvement in the metabolic status of the tissue rather than the restoration of ionic gradient. In this respect it is noteworthy that the depression in the rate of rise of the action potential after cooling is not reversed when the tissue is warmed in high K^+ Ringer.

It has also been reported by Desmedt (7) that there is a lack of correlation between K^+_{in} and resting potential, similar to that found by ourselves. The discrepancies are quite large. For instance, he found that muscles soaked in Ringer containing 0.2 m.eq./liter K^+ had a resting potential of 100 mv., whereas the calculated value would be 156 mv. More important still, this value did not change during 70 hours' soaking, although the K^+_{in} had dropped by 25 per cent, which should have resulted in a fall of 34 mv.

Throughout this work potentials have been calculated by means of the Nernst equation. We have not used the Goldman (4) equation as the constants have not been measured for toad muscle. However, it is possible to discuss some aspects of our results in the light of this equation. For instance, our constancy of the overshoot in the presence of varying amounts of internal Na^+ could be explained by (*a*) a variation in the permeability of Na^+ in high and low Na^+ muscles, but such variation would be reflected in the resting potential, which however, remains constant; (*b*) if the Na^+ permeability remains constant there would have to be an alteration in the permeability of K^+, *i.e.* because the overshoot and resting potential are constant in the presence of large variations in concentration of Na^+ and K^+, the permeabilities of both ions would have to be variable, and dependent on the concentrations. The problem is further complicated because the variations in Na^+ and K^+ concentrations are not related. It is particularly noteworthy that the great variation in the Na^+ ratio (from 1:2 to infinity) between the intracellular Na^+ and the plasma Na^+ has not produced any concomitant variation in the resting potential. If the Goldman equation were correct in predicting the resting potential then as the Na ratio increased there should be a falling off in the resting potential unless the chloride ratio increased at the same time. We have not investigated changes in Cl^- level, but this work is in progress.

The experiment complementary to ours has been carried out by Grundfest

et al. (8), who altered the internal environment of squid axons by microinjection. We have come to conclusions similar to theirs.

If calculations based on the Nernst (or Goldman) equations are unable to account for the potentials observed over a wide range of ionic content in individual muscles, it is necessary to offer some alternative hypothesis to account for bioelectric potentials. We lean to the view of Grundfest *et al.* (8) that one is really concerned with a charged membrane of unknown nature, and the degree of the charged is influenced by the ionic environment. This view has been further strengthened by experiments involving alteration of the external ionic environment, which will be published in a later paper (10).

SUMMARY

The sartorius muscles of 320 toads have been analyzed for Na^+ and K^+. There is a wide variation in the Na^+ content which when calculated intracellularly varied from 0 m.eq./kg. to 58 m.eq./kg. In particular it was found that the distribution of internal Na^+ in the intact animal was such that only 17 per cent of the muscles should give from the Nernst equation the observed overshoot of 37 mv.

In contrast to this wide variation the K^+ content is comparatively constant, the range being 71 to 112 m.eq./kg. The mean observed resting potential of 87 mv. agreed well with the potential calculated from the mean intracellular K^+ by the Nernst equation.

Analyses of plasma show that the Na^+ content is constant at 130 m.eq./liter, and the K^+ is 3.0 m.eq./liter.

The resting and action potentials of 77 muscles have been recorded and then the muscles have been analyzed. The results have shown that there is no correlation between the level of intracellular Na^+ and the overshoot. Furthermore the apparent correlation between the average K^+ content and the *average* resting potential has been shown to be fortuitous, when the correlation in *individual* muscles is considered.

When a muscle is soaked in Ringer solution for several hours there is a gain of Na^+ and a loss of K^+. These shifts should result in changes in the respective potentials, but such changes were not found.

The above findings have been discussed in the light of the present theories that the resting potential and the action potential are directly related to the ionic ratio across the membrane. Our results very definitely do not support the theory that the overshoot is related to the Na^+ gradient, and this also applies with respect to the K^+ gradient and the resting potential.

We wish to thank Mr. George Stecher for his skilled technical assistance. Our thanks are also due to Professor M. Belz and Mr. R. T. Leslie for their advice on some of the statistical procedures.

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CHANGES IN THE DURATION OF THE ELECTRIC RESPONSE OF SINGLE NERVE FIBERS FOLLOWING REPETITIVE STIMULATION

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(Received for publication, January 27, 1956)

Following prolonged repetitive stimulation the properties of the nerve trunk are markedly modified. The amplitude of the action potential is lowered (9, 11, 13), conduction velocity decreased (10), rheobase increased (2), refractory period lengthened (12), after-potential increased (1, 5, 8), and oxygen consumption (7) and heat production (6) per impulse are decreased.

The interpretation of such results is often uncertain because differences exist in the size and form of the action potential, in the excitability, and in the conduction rate, of the fibers comprising the nerve trunk. In the present investigation the effects of tetanization on nerve activity were reexamined using toad single nerve fibers. The most striking changes observed involved the duration of the electric response. This report is mainly concerned with these changes.

Methods

Single nerve fiber responses were recorded with three arrangements of the "bridge insulator" method (14). The nerve innervating the semitendinous or sartorius muscle of the toad (*Bufo marinus*) was used. A segment (about 1 mm. long) of the nerve was desheathed and all except one of the fibers in the desheathed region were cut. The isolated fiber preparation was mounted on a bridge insulator (Fig. 1). The Ringer fluid bathing the preparation was divided into two pools. With the methods illustrated in Figs. 1 *A* and 1 *C* the air gap separating the two pools measured 0.1 to 0.2 mm. With the method illustrated in Fig. 1 *B* the air gap measured 0.5 mm. With all three methods an internode of the isolated fiber was suspended across the air gap. With methods 1 *A* and 1 *B* this internode was located in the dissected region. With method 1 *C* this axis cylinder was located in the undissected portion of the nerve.

The response recorded was derived primarily from the activity of the two nodes adjacent to the internode suspended across the gap. With methods 1 *A* and 1 *B* the response recorded represented the *current* flowing through this internodal segment. When both pools were filled with normal Ringer a binodal action current was obtained. A strong diphasicity or a duration (of the phase of strong current flow) longer than 0.1 to 0.2 msec. was taken as an indication of injury and the fiber was dis-

carded. When the fluid in the distal pool (left in Fig. 1) was replaced with 0.05 to 0.1 per cent cocaine-Ringer, the configuration of the response changed into a triangular form (Fig. 2, record at 0 minutes). This was the response of the node on the proximal side of the air gap (mononodal action current). With method 1 *C* the electrical response

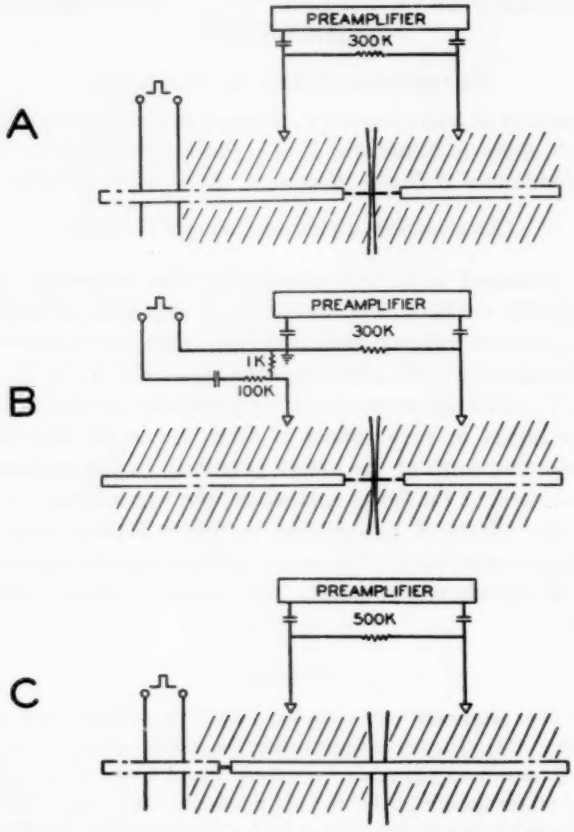

FIG. 1. Three arrangements for recording single nerve fiber responses.

was recorded as *voltage* (15). The configuration of this response was similar to the configuration of the binodal action current.

Tetanic stimuli at 50 to 150 shocks per second were delivered to the fiber for a period of 20 to 140 minutes. Test stimuli were presented at 2 to 5 shocks per second. In most experiments test stimuli were applied continuously following tetanization. In a few experiments a short train of test stimuli was delivered at intervals of 5 minutes to 1 hour. With method 1 *B* the node under study was stimulated directly by passing a current across the two pools. With methods 1 *A* and 1 *C* the node under study was excited indirectly by stimulating the proximal end of the nerve trunk.

In a series of experiments the sciatic plexus of the toad was tetanized *in vivo*. Fol-

lowing tetanization the nerve innervating the sartorius and semitendinosus muscle was excised. Single fiber responses were then studied with methods 1 *A* or 1 *B*.

The accommodation constant (Hill's λ) of single nerve fibers was determined using Tasaki's method (17)

With all three methods the recording electrodes were of the Ag–AgCl–Ringer (agar)

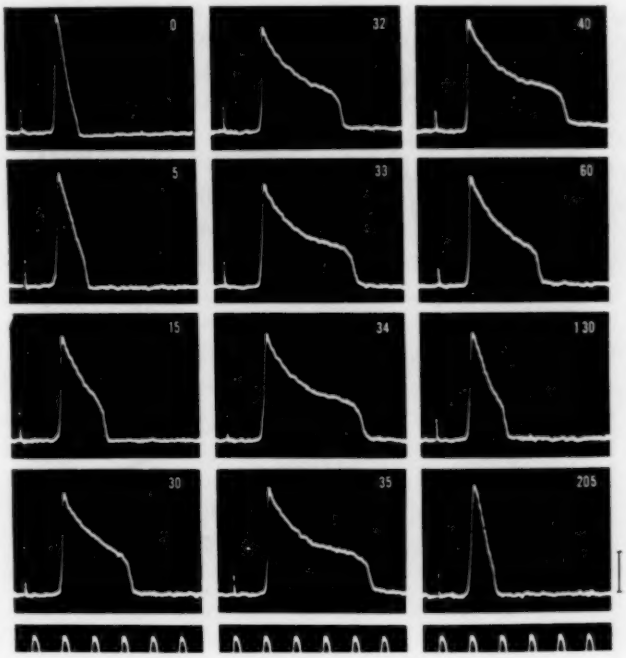

FIG. 2. Action current prolongation during and following 30 minutes' tetanization at 150 shocks per second. The time in minutes from the beginning of tetanization is indicated on the upper right hand corner of each frame. Tetanization was interrupted for a few seconds when tracings were photographed. Test stimuli were delivered at 2 shocks per second. Toad motor nerve fiber. Vertical bar subtends 1×10^{-9} amperes Time marking, 1 K. C. Temperature, 22°C.

type. With methods 1 *A* and 1 *C* the stimulating electrodes were a pair of platinum wires. With method 1 *B* the stimulating electrodes were of the Ag–AgCl–Ringer (agar) type. The instruments employed included a Grass SA4 stimulator, a Tektronix 122 preamplifier, a Dumont 322A dual channel oscilloscope, and a Grass or Beattie-portronic camera. Observations were made on 112 large (10 to 15 μ) motor nerve fibers. The Ringer's fluid contained 0.2 per cent $NaHCO_3$, 0.014 per cent KCl, 0.65 per cent NaCl, and 0.012 per cent $CaCl_2 2H_2O$. In some experiments it contained in addition 0.001 per cent NaH_2PO_4 and/or 0.2 per cent glucose. Experiments were carried out either at room temperature (22–25°C.) or in a refrigerated room (10–13°C.).

RESULTS

The Properties of Untetanized Fibers

At room temperature (22–25°C.) the duration of the mononodal action current was 0.7 to 0.9 msec., the amplitude 2 to 3 $\times$ 10^{-9} amperes, the rheobase (for direct node stimulation) 16 to 25 mv., and the accommodation constant (Hill's λ) 6 to 13 msec. At low temperatures (10–13°C.) the duration was 2 to

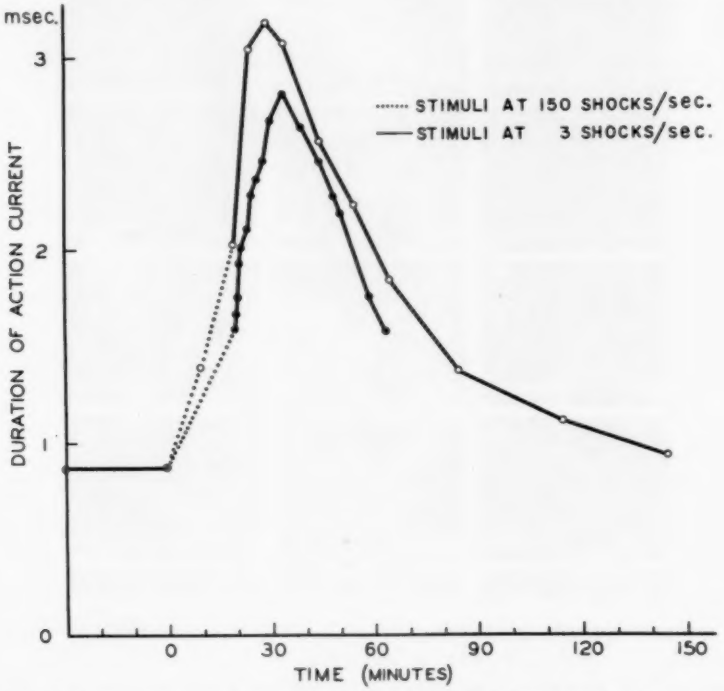

FIG. 3. Action current prolongation during and following tetanization of two toad motor nerve fibers. Temperature, 22°C.

3 msec., the amplitude 1.6 to 2.5 $\times$ 10^{-9} amperes, the rheobase 20 to 25 mv., and the accommodation constant 20 to 25 msec.

The Properties of Tetanized Fibers

During tetanization the spike duration was progressively increased (Figs. 2 and 3). Following tetanization, in 98 out of 112 preparations, the spike duration continued to lengthen at a more rapid rate. Within 5 to 60 minutes the post-tetanic prolongation stopped, and within 1 to 10 hours the spike duration was normal. The duration of the response of tetanized fibers was from 2.5 to more than 10 times the spike duration of untetanized fibers. The dura-

tion of the response of 14 out of 112 fibers was doubled or tripled immediately after tetanization but little or no post-tetanic lengthening occurred.

Post-tetanic spike prolongation was observed with all three methods, 1 *A*, 1 *B*, and 1 *C*, at room temperature and in the cold room, in mononodal and binodal preparations, with continuous or intermittent test stimulation, and with all tetanizing frequencies between 50 and 150 shocks per second. Frequent replacement of the fluid bathing the stimulated node with fresh Ringer during or after tetanic stimulation did not prevent prolongation. Prolongation was observed in fibers isolated from nerves tetanized *in vivo*. Injured fibers (fibers with abnormal binodal spike shape) showed little or no post-tetanic lengthening. With longer periods of tetanization the increase in duration was greater and the time required for the duration to return to normal was longer. The exact dependence of the time course of changes in spike duration upon the frequency and duration of tetanization, the temperature, and other experimental conditions is still under investigation. Large differences among fiber preparations have made such a study difficult.

The rate of fall of the shoulder (the sudden sigmoid ending of the falling phase) of the mononodal action current remained relatively unchanged during and after tetanization (Fig. 2).

In the fibers tetanized after isolation, during tetanization the amplitude of the response was progressively decreased, the rheobase increased, and the shock-response interval prolonged. Tetanized fibers showed a prolonged accommodation constant. In response to D. C. stimulation many tetanized fibers fired repetitively.

After a period of tetanization the fiber failed to respond to every stimulus. This failure occurred more readily with higher frequencies of tetanization. When the frequency of tetanization was decreased the fiber responded again to every stimulus.

DISCUSSION

The changes in amplitude, latency, and rheobase obtained as a result of tetanization with whole nerve trunks (1, 2, 5, 13) are similar to those obtained in the present study with toad single nerve fibers. No evidence, however, exists in the literature for a post-tetanic prolongation of the responses of individual nerve fibers.

Since spike prolongation was obtained *in vivo*, with binodal preparations and with method 1 *C*, prolongation could not have been a result of injury during isolation of the fiber, or (in mononodal preparations) a result of the unidirectional current flowing through the node under study. With method 1 *A* and in the *in vivo* experiments most (if not all) the fibers in the nerve trunk were active during tetanization. This could not have been associated with the prolongation, since prolongation was obtained with method 1 *B*

when the fiber under study was the only fiber in the nerve trunk active during tetanization.

The effects of chemicals on the prolongation following tetanization have recently been reported (16). Certain compounds (*e.g.*, tubocurarine) prevented this prolongation or shortened the prolonged spike. Other chemicals (*e.g.*, versene) further lengthened the duration of the prolonged spike. Versene or tubocurarine had no comparable effects on the untetanized fiber. Reversible post-tetanic prolongation occurred in nitrogen (containing less than 1 per cent O_2), 95 per cent O_2 and 5 per cent CO_2, or in the presence of certain glycolytic or TCA cycle inhibitors.

The time course of the response of tetanized nerve fibers is similar to the time course of the response of cardiac muscle fibers (18), of Purkinje fibers (3), of crustacean muscle fibers treated with TEAC (4), and of nerve fibers treated with NaCl (hypertonic), sinomenine, heroine, brucine, emetine (14), or azide (unpublished experiments).

Prolongation was obtained with stimulating frequencies known to occur in the nervous system. The prolongation during and especially following tetanization cannot easily be explained with any of the existing theories of nerve excitation.

SUMMARY

Single nerve fibers were isolated from the nerve innervating the sartorius or semitendinosus muscle of the toad (*Bufo marinus*). Single nerve fiber responses were recorded with three arrangements of the "bridge insulator" method. During stimulation at 50 to 150 pulses per second for 20 to 140 minutes the spike duration was progressively increased. After tetanization the spike duration usually continued to increase at a more rapid rate. Within 5 to 60 minutes further prolongation stopped and within 1 to 10 hours the spike duration was normal. The duration of the response of tetanized fibers was from 2.5 to more than 10 times the spike duration of untetanized fibers. Prolongation was observed in nerve fibers isolated from nerves tetanized *in vivo*.

The author is indebted to Dr. Ichiji Tasaki for instruction in the single fiber technique and for his help throughout the present study. The author is also grateful to Mrs. N. Tasaki for preparing the figures of the manuscript.

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ALTERATION BY DINITROCRESOL OF PATHWAYS FOR GLUCOSE OXIDATION IN EGGS OF ARBACIA PUNCTULATA

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(Received for publication, February 16, 1956)

Some years ago it was observed that dinitrocresol and other substituted phenols could block the cleavage of fertilized sea urchin eggs in a reversible manner (1–3). These agents have a number of effects on energy-yielding processes which are potentially related to those on cleavage: In low concentrations they inhibit aerobic phosphorylation (4–8), but not the phosphorylation associated with glycolysis (9); they also accelerate breakdown of stored high energy phosphate compounds (10); they inhibit glucose-6-phosphate dehydrogenase of yeast (11) and *Arbacia* eggs (12); and, at rather higher concentrations, they inhibit two flavoprotein enzymes, D-amino acid oxidase (13), and cytochrome reductase (11).

The present paper deals with hitherto unrecognized effects of dinitrocresol which may be pertinent both to its general ability to suppress anabolic and assimilative processes (2, 4, 14–16) and to the ability of substituted phenols to stimulate oxygen consumption. Earlier experiments had indicated that *Arbacia* eggs utilize glucose predominantly *via* the so called hexose monophosphate (TPN) shunt, by which the 1-carbon is preferentially converted to CO_2 (12, 17). In studying the oxidation of glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} by *Arbacia* eggs it has now been found[1] that dinitrocresol apparently diverts glucose oxidation from the TPN shunt pathway into another pathway, presumably that *via* glycolysis and the tricarboxylic acid cycle, in which the 6- , 2- , and 1-carbons of glucose are transformed to $C^{14}O_2$ at comparable rates. These observations are of interest in relation to the suggestion that various regulatory factors, including hormones, may act in part by diverting metabolites from one pathway into another (27, 28).

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[1] A preliminary account of this work was presented in abstract form at the General Scientific Meeting of the Marine Biological Laboratory, August, 1955 (29, 30).

Experimental Methods and Materials

Eggs were obtained and handled as previously described (17, 18). Formation of $C^{14}O_2$ and other products was measured over a 2 hour period for eggs at three stages: (*a*) unfertilized; (*b*) beginning 25 minutes after fertilization; (*c*) beginning 24 hours after fertilization and development at 25°C., when the eggs were swimming plutei (19). Incubation was carried out in Warburg vessels at 20°C. The main compartment received 4 ml. of a suspension of 2 per cent eggs (by volume) in sea water or in solutions of dinitrocresol in sea water at pH 8, as shown in the tables; the center well contained 0.5 ml. 0.3 N NaOH, the side arm, 0.5 ml. 0.1 N HCl. The flasks were shaken for 2 hours, after which the HCl was tipped from the side arm into the main compartment; shaking was continued for 60 minutes to displace CO_2 into the center well. The $BaC^{14}O_3$ and other fractions were isolated, and their radioactivity measured, as described elsewhere (17).

The *total* glucose concentration in the sea water of the incubation medium was 0.0006 M in every experiment. Each radioactive sugar was diluted with an appropriate amount of non-labelled glucose so that the contents of one incubation flask, 4 ml. of 2 per cent egg suspension, contained 644,000 C.P.M. at the beginning of incubation. The specific activity of the glucose was 270,000 C.P.M. per micromole under the counting conditions employed.

All results are expressed in terms of the changes occurring in a single incubation flask over a 2 hour period at 20°C. Each flask contained 80 c.mm. of eggs or embryos, having a wet weight of approximately 88 mg. and a dry weight of about 21 mg., of which about 14 mg. was protein (18); the number of eggs per flask was approximately 380,000.

The purchase of the labelled sugars was made from a grant-in-aid by the Atomic Energy Commission to the Marine Biological Laboratory.

RESULTS

Two concentrations of dinitrocresol (DNC) were tested; the one, 8×10^{-6} M, is that which gives optimum stimulation of oxygen consumption in *Arbacia* eggs and reduces the rate of cleavage of eggs and the aerobic phosphorylation in egg extracts by some 50 per cent; the other concentration, 1.28×10^{-4} M, is above that for optimum respiratory stimulation and blocks cleavage and aerobic phosphorylation completely (2, 8).

Glucose oxidation to $C^{14}O_2$ (Table I).—The control values for normal eggs and embryos illustrate the fact, discussed in detail elsewhere (17), that $C^{14}O_2$ production from glucose-1-C^{14} was very much more rapid than from glucose-2-C^{14} or glucose-6-C^{14}. The ratios of $C^{14}O_2$ from glucose-2-C^{14} and from glucose-6-C^{14} to that from glucose-1-C^{14} were, respectively: for unfertilized eggs, 0.32 and 0.06; for eggs fertilized 25 minutes before start of incubation, 0.59 and 0.17; for 24 hour embryos (average of two experiments), 0.47 and 0.33.

Dinitrocresol produced an increase in $C^{14}O_2$ formation from glucose-6-C^{14}; with 8×10^{-6} M DNC the total radioactivity collected as $BaC^{14}O_3$ was raised from 340 to 9,400 C.P.M. for unfertilized eggs, from 570 to 3,200 for eggs fertil-

ized 25 minutes, and from 18,000 to 67,000 for 24 hour embryos (Experiment 21 W, see also 28 W). There was apparently a concomitant inhibition of the original preferential pathway for carbon-1 to $C^{14}O_2$: if it is assumed that the new pathway transforms the 1- carbon to $C^{14}O_2$ at the same rate as the 6-carbon, it may be calculated that in 8×10^{-6} M DNC the residue of the original pathway for unfertilized eggs is 11,300 − 9,400 = 1,900 as compared to the control of 5,900 − 340 = 5,560. Similarly, for the fertilized eggs the respective values

TABLE I

Effect of Dinitrocresol (DNC) on Oxygen Consumption and $C^{14}O_2$ Formation from Glucose-1-C^{14}, Glucose-2-C^{14}, and Glucose-6-C^{14} by Arbacia Eggs and Embryos during 2 Hours of Incubation at 20°C.

Each sample contained 88 mg. eggs or embryos wet weight. For details of technique, see Experimental Methods.

Experiment No.	Date	State of egg development at start of incubation	Concentration DNC	O_2 consumed	Total c.p.m. into $BaCO_3$ from:				
					Glucose-1-C^{14}	Glucose-2-C^{14}	g-2-C^{14} / g-1-C^{14}	Glucose-6-C^{14}	g-6-C^{14} / g-1-C^{14}
	1955		*moles per liter*	*c.mm.*					
20W	7–26	Unfertilized	0	12	5900	1900	0.32	340	0.06
			8×10^{-6}	76	11,300	12,300	1.08	9400	0.83
			1.28×10^{-4}	31	6600	4700	0.71	1600	0.24
19W	7–25	Fertilized 25 min.	0	55	3400	2000	0.59	570	0.17
			8×10^{-6}	139	4800	4400	0.92	3200	0.67
			1.28×10^{-4}	63	4500	4000	0.89	2200	0.49
21W	7–28	24 hr. embryos	0	143	52,000	24,000	0.46	18,000	0.35
			8×10^{-6}	272	83,000	87,000	1.05	67,000	0.81
			1.28×10^{-4}	211	88,000	98,000	1.10	82,000	0.93
28W	8–12	24 hr. embryos	0	119	40,000	19,000	0.47	12,000	0.30
			8×10^{-6}	218	52,000	43,000	0.83	40,000	0.77
			1.28×10^{-4}	178	64,000	68,000	1.06	62,000	0.97

are: residue, 1,600, control, 2,830; for embryos (Experiment 21 W): residue, 16,000, control, 34,000.

The effects with 1.28×10^{-4} M DNC were more complex, but at each stage of development there was an apparent inhibition of the pathway for preferential oxidation of carbon-1 of glucose.

The 8×10^{-6} M dinitrocresol also produced an increase in $C^{14}O_2$ formation from glucose-2-C^{14}; this increase was either not significantly different from, or slightly larger than, the increase in $C^{14}O_2$ from glucose-6-C^{14}. The possible significance of these observations will be discussed below.

The question arose to what extent the DNC-induced increase in $C^{14}O_2$ from

glucose-C^{14} might be due to an increase in utilization of glucose from the medium, especially as DNC has been found to increase glucose utilization by ascites tumor cells (20). Accordingly, measurements of glucose uptake and glycogen breakdown were made. Glucose uptake from the medium by unfertilized eggs was approximately doubled (Table II) by 8×10^{-6} M DNC, while the $C^{14}O_2$ from glucose-6-C^{14} was increased 28 times (Table I); the glucose uptake by the embryos was not increased at all, while $C^{14}O_2$ from glucose-6-C^{14} was increased 3.5 times. Thus the effect of 8×10^{-6} M DNC on $C^{14}O_2$ formation was not attributable solely to change in glucose uptake.

TABLE II

Consumption of Glucose from Medium and from Egg Polysaccharide (Glycogen) during 2 Hours of Incubation at 20°C.

The quantities of eggs and other conditions were the same as in Table I.

Experiment No.	Date	State of egg development at start of incubation	Concentration DNC	Glucose consumed, μg.	
				From medium	From egg polysaccharide
	1955		*moles per liter*		
24W	8–2	Unfertilized	0	68	157
			8×10^{-6}	118	236
			1.28×10^{-4}	100	162
23W	8–1	24 hr. embryos	0	91	235
			8×10^{-6}	77	287
			1.28×10^{-4}	90	235

No glucose was liberated into the medium during incubation of eggs or embryos without added glucose. No lactate was formed either without or with added glucose in the medium.

Incorporation of C^{14} from Labelled Glucose into other Fractions (Table III).— Both concentrations of DNC reduced incorporation of C^{14} from glucose-C^{14} into the acid-insoluble fraction, which contained the nucleoproteins and some of the lipides of the eggs (Table III). This was consistent with the now well known suppression of anabolic effects in a number of types of cells (14–16, 21)

There were inhibitory effects upon transformation of glucose-C^{14} to other products (Table III). These effects were especially clear-cut for the embryos, where transformation of glucose-C^{14} to all products proceeded in the controls at a much greater rate than in the unfertilized and just fertilized eggs.

DISCUSSION

Arbacia eggs contain a number of the enzymes for utilization of glucose *via* the glycolytic pathway and the tricarboxylic acid cycle (12, 22). They also contain at least four of the enzymes, hexokinase (23), glucose-6-phos-

TABLE III

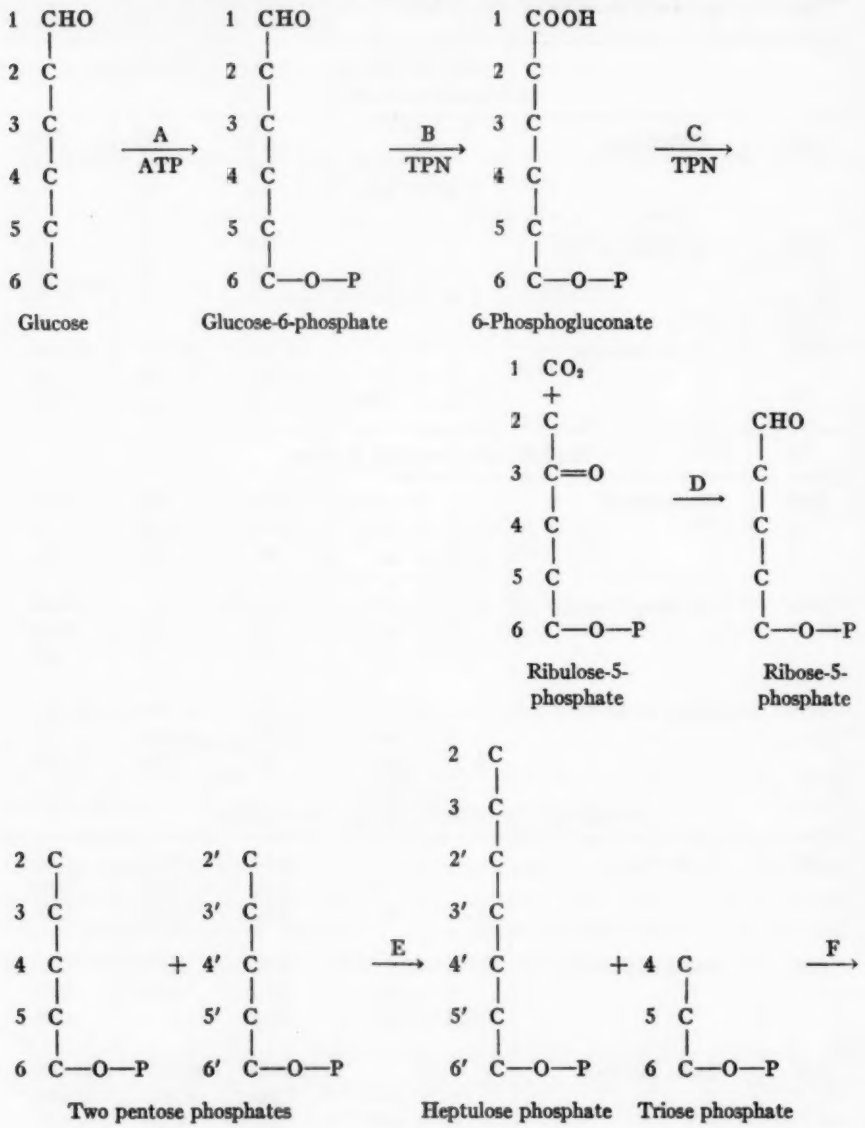
Effects of Dinitrocresol on Incorporation of Radioactivity from Glucose-1-C^{14}, Glucose-2-C^{14}, or Glucose-6-C^{14} into Acid-Insoluble Fraction, Barium Salts Insoluble in Water, and Barium Salts Insoluble in 80 Per Cent Ethanol

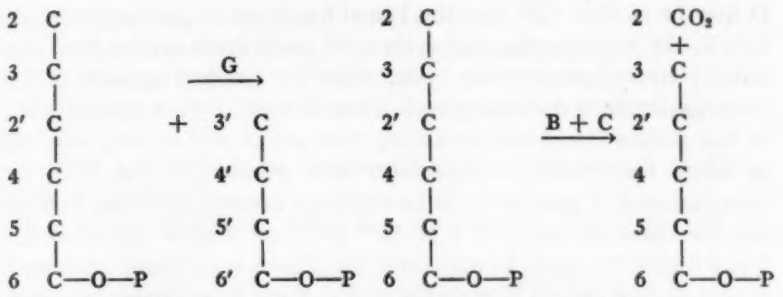
The experiments are the same as those in Table I.

Experiment No.	State of egg development at start of incubation	Concentration DNC	Total c.p.m. from:		
			Glucose-1-C^{14}	Glucose-2-C^{14}	Glucose-6-C^{14}
		moles per liter			
Acid-insoluble fraction					
20W	Unfertilized	0	1300	1700	1400
		8×10^{-6}	300	200	200
		1.28×10^{-4}	60	60	50
19W	Fertilized 25 min.	0	300	300	300
		8×10^{-6}	200	100	200
		1.28×10^{-4}	50	60	50
21W	24 hr. embryos	0	32,000	49,000	42,000
		8×10^{-6}	15,000	18,000	17,000
		1.28×10^{-4}	1600	1400	1600
Barium salts insoluble in water					
20W	Unfertilized	0	4000	5800	5300
		8×10^{-6}	3200	3400	4100
		1.28×10^{-4}	2800	3100	2800
19W	Fertilized 25 min.	0	1300	2300	1900
		8×10^{-6}	900	1200	1100
		1.28×10^{-4}	1200	2100	1600
21W	24 hr. embryos	0	30,000	35,000	35,000
		8×10^{-6}	17,000	24,000	24,000
		1.28×10^{-4}	7900	7700	16,000
Barium salts insoluble in 80 per cent ethanol					
20W	Unfertilized	0	6000	9500	8000
		8×10^{-6}	2400	3800	2800
		1.28×10^{-4}	3800	5300	4600
19W	Fertilized 25 min.	0	4000	8000	7800
		8×10^{-6}	3800	4600	3800
		1.28×10^{-4}	3500	—	8400
21W	24 hr. embryos	0	25,000	40,000	42,000
		8×10^{-6}	7200	16,000	32,000
		1.28×10^{-4}	7200	8600	14,000

phate dehydrogenase (12), 6-phosphogluconate dehydrogenase (12), and hexose phosphate isomerase (23), of the TPN shunt pathway by which the 1-carbon of glucose is transformed preferentially to CO_2 and pentoses are formed. One current version of the shunt pathway is (24):

Pathway for Glucose Oxidation via TPN Shunt





Fructose-6-phosphate Tetrose phosphate Glucose-6-phosphate Pentose phosphate

The reactions are: A, hexokinase; B, glucose-6-phosphate dehydrogenase; C, 6-phosphogluconate dehydrogenase; D, pentose phosphate isomerase; E, transketolase; F, transaldolase; G, hexose phosphate isomerase.

According to this pathway the 1-carbon appears as CO_2 when a single glucose molecule has undergone steps A, B, and C; the 2-carbon appears as CO_2 after oxidation of *two glucose molecules* through step G and the repetition of steps B and C; the 6-carbon appears as CO_2 only if the triose phosphate, the tetrose phosphate, or the hexose phosphates reenter the glycolytic cycle at the appropriate points.

From the standpoint of conversion of glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} to $C^{14}O_2$ the important difference between the glycolytic pathway and the TPN shunt pathway is that the former provides two triose phosphate moieties in which the 1- and the 6-carbons enter the pathway toward CO_2 equally rapidly; the TPN shunt pathway, on the other hand, transforms the 1-carbon directly to CO_2 and provides a triose phosphate molecule with no labelling from the 1-position.

It is of interest to consider the results with dinitrocresol in Tables I and III in relation to the known enzymatic events in *Arbacia* eggs and the effects thereon of substituted phenols. The principal finding in Table I is that 8×10^{-6} M dinitrocresol increases $C^{14}O_2$ production from glucose-6-C^{14} relative to that from glucose-1-C^{14}. It is known that substituted phenols inhibit the enzymes for steps B and C in extracts of *Arbacia* eggs (12). This would tend to increase the steady state concentration of glucose-6-phosphate and, in turn the concentration of fructose-6-phosphate and subsequent substrates of the glycolytic cycle. Such a sequence of events favors utilization of glucose-6-phosphate *via* the glycolytic pathway, in which the 1- and 6-carbons appear equally rapidly as CO_2. This may explain, in part at least, the relative increase in $C^{14}O_2$ from carbon-6.

There is another observation in Table I to be accounted for, namely, the finding that the increase in $C^{14}O_2$ from glucose-2-C^{14} produced by 8×10^{-6} M DNC is sometimes larger than the increase in $C^{14}O_2$ from glucose-6-C^{14}.

It may be recalled (25) that the 1- and 6-carbons of glucose do not appear as CO_2 in the tricarboxylic acid cycle until oxalacetate arising from the citrate initially formed reenters the cycle, while the 2-carbon appears as CO_2 when α-ketoglutarate is decarboxylated. Thus, if there were a relatively large pool of four carbon intermediates, arising from amino acid or fatty acid oxidation, to dilute the labelled intermediates from glucose-C^{14}, the $C^{14}O_2$ formation from carbon-2 of glucose would be relatively favored over that from carbon-6, and the observed effects of 8×10^{-6} DNC on relative oxidation of carbons 2 and 6 to $C^{14}O_2$ could be accounted for. There is no direct evidence for such a pool of four carbon intermediates, but there is evidence that amino acids and fatty acids contribute substantially to the total oxidative metabolism of *Arbacia* eggs (18, 26).

The reduction of C^{14} incorporation into the other fractions, which is more or less independent of the position in which the glucose is labelled, may be provisionally interpreted as a consequence of inhibition, by the dinitrocresol, of ATP production *via* aerobic phosphorylation in the eggs (8); the proportionate reductions in synthesis ensuing from the lowered steady state ATP concentration vary according to the concentrations of ATP required to saturate the various synthetic enzymes in question and according to the quantitative energy requirements of the cells at the various stages of development.

The authors wish to thank Miss Patricia Walters for her cooperation.

SUMMARY

1. The $C^{14}O_2$ production by *Arbacia* eggs and embryos from glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} has been measured without and with dinitrocresol in the incubation medium. In the absence of the dinitrocresol, the $C^{14}O_2$ production from glucose-1-C^{14} is more rapid than from glucose-2-C^{14} and much more rapid than from glucose-6-C^{14}; this, together with previous findings, indicates that glucose is utilized in *Arbacia* eggs predominantly *via* the TPN shunt rather than *via* the aldolase step of the glycolytic pathway. In the presence of the dinitrocresol, $C^{14}O_2$ from glucose-6-C^{14} approaches that from glucose-1-C^{14}, indicating that, in the presence of this reagent, glucose utilization is diverted from the shunt to the glycolytic pathway.

2. Incorporation of C^{14} from glucose labelled in the 1-, 2-, or 6- positions into other metabolic products of the eggs and embryos is also inhibited by dinitrocresol, particularly incorporation into the acid-insoluble fraction containing nucleoproteins.

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THE INHIBITION OF HEMOLYSIS, AS STUDIED BY THE TECHNIQUE USED FOR INVESTIGATING PROGRESSIVE REACTIONS, AND BY A TECHNIQUE USING RADIOACTIVE HEMOLYSINS*

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(Received for publication, January 23, 1956)

Our understanding of inhibition and acceleration of hemolysis is still in an unsatisfactory state. One possibility is that an inhibitor reacts with a lysin to form a relatively non-lytic compound or complex, as when cholesterol reacts with digitonin to form the almost insoluble cholesterol digitonide. Such a reaction (termed a Δ reaction) would decrease the concentration of lysin in the system, and lysis would be correspondingly slower. Another possibility is that the inhibitor reacts with the red cell surface making it more (or less) resistant to a reaction with the lysin; this possibility is particularly attractive when the system contains, not an inhibitor, but an accelerator, and there are instances in which reactions of this kind (termed R reactions) can be demonstrated directly (Ponder, 1939).

In investigating Δ reactions and R reactions it is usual to mix the lysin with the inhibitor or the accelerator, and then to add the red cells. The times for complete hemolysis in different concentrations of the lysis, without and with the addition of the inhibitor or accelerator, are measured; if a concentration of lysin c_1 to which inhibitor has been added takes the same time to produce complete hemolysis as a smaller concentration c_2 to which the inhibitor has not been added, the amount of lysin rendered inert in a Δ reaction is $\Delta = c_1 - c_2$, while the increase in resistance of the cells in an R reaction is $R = c_1/c_2$. If the values of R are less than 1.0, there is acceleration instead of inhibition; in the case of acceleration of hemolysis, however, Δ has no clear meaning.

This simple method of approaching the problem has been very useful, but it rests on at least three assumptions. The first is that the reaction between the lysin and the inhibitor is substantially irreversible, the second is that the system surrounding the red cells is spatially homogeneous, and the third is that hemolysis is the result of a simple one stage process in which the lysin reacts with components of the red cell structure. For most purposes, the first assumption has seemed to be valid, although there are effects, involving the variation in

* This investigation was supported by a grant, H 1598, from the United States Public Health Service.

the amount of lysin rendered inert by serum when the number of red cells in the system is varied, which have led to attempts to introduce some degree of reversibility (Ponder and Gordon, 1934). The second and third assumptions are no longer tenable since the demonstration that hemolytic reactions may be *progressive* (Ponder and Cox, 1952, Ponder, 1953), and since the demonstration that there may be a rapid uptake of lysin at the red cell surfaces to form an "internal lysin phase" (Croes and Ruyssen, 1951 *a*, 1951 *b*, Ponder and Ponder, 1954). The evidence now points to lysis in some systems containing simple hemolysins being a process involving two stages in time and two lysin phases. The first state in time is the entry of the lysin into an "internal lysin phase" situated at the cell surfaces, a process which may be partially reversible at first, and which may be referred to as "fixation" of the lysin. The second stage is the irreversible reaction of the fixed lysin in the "internal lysin phase" with the cell components, and this is the hemolytic reaction proper.

In view of these developments, it is desirable to investigate inhibitory phenomena by the same methods as have established that lytic reactions may be progressive and that there may be an internal lysin phase in the neighborhood of the red cell surfaces, particularly as such investigations show that there are inhibition-producing mechanisms in addition to those already considered. This paper will deal primarily with the inhibitory effects of human plasma, but some notes on the effects of lecithin, cholesterol, and serum albumin will be added.

Technique

The method used for showing that some hemolytic reactions are progressive consists in allowing the reaction to proceed until a certain percentage, P_0, of complete hemolysis has occurred, and at this moment diluting the system with sufficient saline (usually 10 volumes) to render the concentration of lysin in the system less than asymptotic. If lysis continues from P_0 to $(P_0 + g)$ in spite of this dilution, the process is progressive and there is a method (Ponder, 1953) by means of which the extent of the dilution of lysin in the internal phase can be calculated; in experiment, the dilution of the system as a whole is usually 10-fold, and the calculation yields the result that the dilution of the lysin in the internal phase is F-fold. Except in some special cases, $F = <10$ in saponin systems; when the lysin in the internal lysin phase is completely unaffected by the addition of the saline, $F = 1.0$ (systems containing digitonin and the bile salts).

Instead of adding saline to dilute the system when P_0 has reached a certain value, an inhibitor can be added, and F can be calculated as before. If the inhibitor is without effect on the lysin in the internal phase, $F = 1.0$, whereas if it reacts with the lysin in the internal phase to produce inhibition there, F may attain considerable values.[1] Except that an inhibitor, instead of a large volume of saline, is added to the

[1] The values reached, however, are never as large as the values of R obtained when plasma is added to the lysin, the cells being added afterwards. There is a cer-

system when P_0 has a certain value (*e.g.*, $P_0 = 20$, $P_0 = 40$, etc.), the technique is similar to that already described (Ponder, 1953). Specifically, the systems consist of 1 ml. of lysin in various concentrations, 0.5 ml. of a standard red cell suspension (1.25×10^8 cells), and inhibitor contained in 0.5 ml. of saline. The quantity of inhibitor is denoted by I, the undiluted material, *e.g.* plasma, being one for which $I = 1.0$; thus, when the inhibitor is plasma, $I = 0.1$ means that the 0.5 ml. of inhibitor added to the system consists of plasma diluted 1 in 10, $I = 0.02$ means that the 0.5 ml. of added inhibitor consists of plasma diluted 1 in 50, and so on. In the calculations, F is always

$$\frac{\text{Initial concentration of lysin } c_1}{\text{Concentration of lysin which gives } (P_0 + g) \text{ per cent lysis}}$$

In the case of saponin, g was measured after 10 minutes, and the denominator of the expression for F was taken from a percentage hemolysis curve similar to that in Ponder, 1953, Fig. 3 a, but in which the percentage hemolysis in various lysin dilutions was measured after 10 minutes. In the case of digitonin, a curve showing the percentage hemolysis after 2 hours was used instead, and g was measured at the end of 2 hours. All the experiments were carried out at 25°C.

Since the technique is complicated and since there are so many combinations of conditions which could be considered, the description of the experimental results will be confined to those which throw new light on what is known about inhibitory phenomena.

1. Saponin and Plasma[2]

(*a*) P_0 *Constant*, c_1 *constant* (*two values*), I *Varying*.—Table I, upper part, shows values of g and of F obtained when $P_0 = 20$, when $c_1 = 100\ \gamma$, and when I varies from zero to 0.10. The progressive nature of the reaction, measured by g, becomes less as I increases; this means that if lysis is allowed to proceed to 20 per cent hemolysis before the inhibitor is added, the further progress of the reaction is inhibited more and more as the quantity of added inhibitor is increased. Fig. 1 results from plotting F against I. When $I = 0$, $F = 1.0$, *i.e.* there is no inhibition, and as I increases F increases, at first sharply and then more slowly, towards a limiting value. This value is reached when $P_0 = 20$

tain similarity between F and R, as can be seen from their definitions. F is the extent to which a system containing a concentration c_1 of lysin must be diluted to give a concentration c_x which provides just enough lysin for $(P_0 + g)$ per cent hemolysis; R (measured at the asymptotes) is the extent to which a system containing a concentration c_1 of lysin must be reduced in concentration to give the concentration c_{100} which provides just enough lysin for 100 per cent hemolysis.

[2] Neither lecithin (500 γ) nor cholesterol (100 γ) prevents the lytic reaction from progressing, even if these inhibitors are added to saponin-cell systems in which P_0 is as small as 20. In lysin-inhibitor-cell systems, on the other hand, 500 γ of lecithin gives an R value of about 1.5, while as little as 10 γ of cholesterol gives an R value of 2.0.

$g = 0$; $(P_0 + g) = 20$, γ for $P = 20 = 55\ \gamma$, and $100/55 = 1.8$, the value of F for complete inhibition. A rough extrapolation shows that this limit is reached when I is about 1.5. A similar relation between F and I is found when $c_1 = 67\ \gamma$ (dotted line in Fig. 1). Here the limiting value of F is 1.22.

(*b*) c_1 *and* I *Constant*, P_0 *Varying*.—The results shown in the lower part of Table I were obtained with systems in which $c_1 = 100\ \gamma$ and $I = 0.1$, P_0 being varied from 5 to 55. The value of g increases as P_0 increases; *i.e.*, as the lysin becomes more and more firmly attached to the cell surfaces. The values of F vary from 2.0 when $P_0 = 5$ to 1.2 when $P_0 = 55$, and the relation between P_0, c_1, and g is quite similar to that found when lysin-cell systems are diluted

TABLE I

$c_1 = 100$, $P_0 = 20$				
I	g	$(P_0 + g)$	γ for $(P_0 + g)$	F
0.0	80	100	100	1.0
0.01	65	85	77	1.3
0.02	25	45	66	1.5
0.05	15	35	62	1.6
0.10	8	28	59	1.7
$c_1 = 100$, $I = 0.1$				
P_0	g	$(P_0 + g)$	γ for $(P_0 + g)$	F
5	5	10	50	2.0
15	13	28	59	1.7
25	20	45	66	1.5
35	30	65	70	1.4
55	40	95	85	1.2

with large volumes of saline (Ponder, 1953, Fig. 2). It should be noticed that the relation is quite different from that found with digitonin-cell-plasma systems (Section 2, below).

These limiting values, corresponding to complete inhibition of the progressive reaction, are much smaller than the values of R which would be obtained if the lysin were to be mixed with the inhibitor and the cells added afterwards. This means that the fixation of lysin to the cell surfaces during the time required for 20 per cent hemolysis results in some of the lysin being no longer subject to the inhibitory action of the inhibitor, presumably because it is already fixed with relation to the cell components. Because it is fixed, the reaction progresses; there might, however, be a quantity of lysin at the cell surfaces, not fixed in the same sense as the foregoing but which would become fixed at a later stage of the reaction, and it is possible that this quantity might be removed from the cell surfaces as a result of a combination with the inhibitor, but without the progressive reaction being affected (see Section 4,

which gives an answer to the question: In a system in which some of the lysin has formed an internal lysin phase at the cell surfaces, does the addition of an inhibitor result in some of the lysin being displaced into the bulk phase?).

2. Digitonin and Plasma

When tested by the method which dilutes them, systems containing digitonin are progressive even if diluted when $P_0 < 5$; $F = 1.0$, and the extent to which the lytic reaction progresses seems to be virtually unaffected by the dilution, so firmly and so quickly is the lysin attached to the cell surfaces (Ponder, 1953).

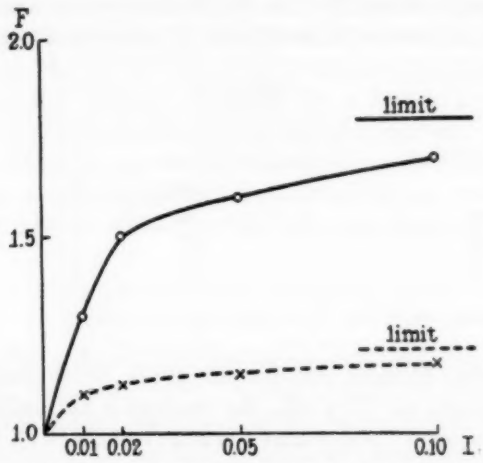

FIG. 1. Ordinate, inhibitory effect of plasma in the internal lysin phase, measured by F; abscissa, quantity of plasma added, measured by I. Full curve, 100 γ of saponin; dotted curve, 67 γ of saponin. For further explanation, see text.

When plasma is added to systems containing digitonin, the results again point to the lysin being firmly and rapidly attached to the cell surfaces. Using enough digitonin to produce complete hemolysis and plasma diluted 1 in 10 ($I = 0.1$) we find that when $P_0 = 5$, $g = 95$, when $P_0 = 20$, $g = 80$, and so on, $(P_0 + g)$ being always approximately 100 and F being always nearly 1.0. It is true that the added plasma slows the hemolytic process, and so it may be inferred that it affects the reaction in some way as yet undefined[3], but it is

[3] Why both dilution and the addition of an inhibitor reduces the velocity of lysis in digitonin systems but leaves the final effect unaffected is a point which is still obscure. Perhaps there is a continual uptake of lysin from the bulk phase as the reactions in the internal lysin phase proceed, and the inhibitor may render the lysin in the bulk phase relatively inactive, thus slowing the hemolytic reaction while still permitting it to go to completion. Perhaps the inhibitor itself enters the internal

almost without effect on the final result; digitonin, once allowed to come into contact with the cell surfaces rapidly forms an internal lysin phase (Ponder and Ponder, 1954), and the lysin in this phase is so firmly attached that its final effect is almost unaffected either by dilution or by the addition of plasma.[4]

3. Sodium Lauryl Sulfate and Plasma

This lysin was selected for the study of inhibitory effects because it can be prepared in a radioactive form (Croes and Ruyssen, 1951 *a*, 1951 *b*). It produces complete hemolysis of 0.5 ml. of a standard human red cell suspension in a concentration of about 50 γ/ml., while the concentration which produces only a trace of lysis is about 25 γ/ml. In the latter concentration, the reaction is not progressive, dilution with 10 volumes of saline not being followed by any

TABLE II

I	g	F
0.0	60	1.0
0.01	30	1.15
0.02	25	1.20
0.10	20	1.25

$P_0 = 40$ in all cases.

additional hemolysis (Ponder and Ponder, 1954). When plasma is added to higher concentrations, *e.g.* 30 γ/ml., the reaction is not progressive provided that P_0 is small enough (<20) or that I is large enough (0.1), but there is some

lysin phase, reducing velocity constants of the reactions which take place there, and so slowing the reaction. The answer to such speculations will no doubt be forthcoming when radioactive saponin and digitonin are available. Meantime the observation that the progress of reactions in systems containing saponin or digitonin is scarcely affected, even with respect to velocity, when a suspension of ghosts (ρ = 0.02) is added when P_0 = 10, 20, etc., suggests that the latter possibility is more important than the former. The inhibitory components of plasma would be expected to enter the internal lysin phase more readily than would large objects such as ghosts.

[4] The addition of 0.2 ml. of a cholesterol sol (100 γ of cholesterol, Lee and Tsai, 1942) appears to be a little more inhibitory than plasma with the same cholesterol content, values of F between 1.2 and 1.4 resulting. As in all digitonin systems, however, the value of g depends on how long one waits to measure it. By contrast, 15 γ of the same cholesterol sol, if allowed to remain in contact with 10 γ of digitonin for 24 hours, gives an R value of 1.8; *i.e.*, cholesterol allowed to stand together with digitonin is about 20 times more effective as an inhibitor than it is when it is added after the lysin has been in contact with the cells. Lecithin (500 γ) has virtually no inhibitory effect on digitonin hemolysis if added to the lysin-cell system, even when P_0 is as small as 10.

progression if the quantity of added plasma is insufficient or if P_0 is large. In still higher concentrations, the reaction is definitely progressive, g depending on P_0 and on I; *e.g.*, when the system contains 40 γ/ml. of lysin, we have the values in Table II.

If the lysin is mixed with the inhibitor before the addition of the cells, the inhibition observed is considerable; *e.g.*, when 50 γ/ml. of lysin is mixed with 0.1 ml. of plasma contained in 0.5 ml. of saline, R = 4.0, and when the same amount of lysin is mixed with 0.02 ml. of plasma, R = 1.8.

These systems are difficult to work with, partly because lysis is so rapid in systems which produce complete lysis, and partly because of special features, involving a tendency for lysis to stop before it is complete (Love, 1950, but see also a criticism by Hutchinson, 1955). When all allowances have been made for these difficulties and for the uncertainties which result from them, the way in which sodium lauryl sulfate is inhibited by plasma is similar, on the one hand, to what occurs in systems containing saponin, red cells, and added plasma, *i.e.*, the hemolytic reaction is progressive if c_1 or P_0 is large enough, or if I, the quantity of added inhibitor, is small enough; on the other hand, the systems resemble systems containing digitonin because of the small values of F obtained.

4. Sodium Lauryl Sulfate-S^{35} and Plasma

It has already been shown that the radioactive hemolysins, sodium lauryl sulfate-S^{35}, sodium cetyl sulfate-S^{35}, and sodium lauryl sulfonate-S^{35} are taken up at the surfaces of washed red cells so as to form an internal lysin phase which may contain as much as 40 per cent of the lysin present before the cells were added (Croes and Ruyssen, 1951 *a*, 1951 *b*; Ponder and Ponder, 1954). These radioactive lysins can be used to answer two questions: (*a*) If an inhibitor is added to the lysin, is the uptake of the lysin by *subsequently* added red cells interfered with?; (*b*) If an inhibitor is added to a cell-lysin mixture in which the lysin has already formed an internal lysin phase at the cell surfaces, is the distribution of lysin between the bulk phase and the internal lysin phase modified?

The greatest concentration of sodium lauryl sulfate-S^{35} to which a washed human red cell suspension of volume concentration 0.3 can be added without lysis occurring was first determined; the dilution of lysin was about 1 in 50,000. Systems were then prepared as follows, the lysin being diluted 1 in 50,000, and the plasma being diluted 1 in 2:

A. 2 ml. lysin + 0.7 ml. saline
B. 2 " " + 0.3 " cell suspension, $\rho = 0.3$
C. 2 " " + 0.5 " plasma + 1 ml. cell suspension added 3 minutes later
D. 2 " " + 1 " cell suspension + 0.5 ml. plasma added 3 minutes later

On completion, each system was centrifuged and the supernatant fluid was removed. Only a trace of lysis occurred. Samples, 0.2 ml. in volume of each supernatant fluid, were placed on planchettes and dried. The radioactivity of each sample was found by Geiger-Müller counting with a thin walled GM tube, or with a windowless flow counter operating in the GM region. A correction was applied for the greater volume of systems C and D as compared with systems A and B. Table III shows typical results.

The conclusions which can be drawn from these results are the following. (*a*) There is an uptake of lysin when red cells are added to 1 in 50,000 sodium lauryl sulfate-S^{35}, the lysin in the bulk phase being about one-sixth of the lysin present in the system at the time the cells were added. (*b*) If plasma (either undiluted, diluted 1 in 2, or diluted 1 in 10) is present *before* the addition of the cells to the lysin, the concentration of the lysin in the internal lysin phase is greatly reduced. (*c*) If plasma (in the same dilutions) is added *after* the cells have been added to the lysin, and presumably after an internal lysin phase has

TABLE III

System	Corrected counts, bulk phase
A	121
B	17
C	73
D	84

been established, the distribution of lysin between the internal phase and the bulk phase is greatly modified, the concentration of lysin in the internal phase again being greatly reduced. In system C (conclusion (*b*) above) the presence of inhibitor tends to prevent the lysin, with which it can readily combine, from becoming fixed at the red cell surfaces. In system D (conclusion (*c*), above) there seems to be a competition for lysin between the cell surfaces and the added inhibitor, the result being that little excess of lysin remains in the internal phase. Similar systems in which the plasma was diluted 1 in 10 show similar results, but the more dilute plasma is a little less effective in preventing the lysin from forming an internal phase, and a little less effective in competing with the cell surfaces for lysin.[5, 6]

[5] If 2 per cent human serum albumin is used as an inhibitor instead of plasma diluted 1 in 2, the results obtained are very similar to those shown in Table III. The prevention of lysin uptake at the red cell surfaces and the redistribution of lysin which occurs when the albumin is added after the internal phase has been formed are accordingly brought about by serum albumin in much the same way as they are brought about by plasma itself.

[6] Virtually the same results have been obtained by using sodium cetyl sulfate-S^{35}, plasma, and albumin.

It should be noticed that $P_0 = 0$ in these experiments, *i.e.* lysin has formed an internal lysin phase (*cf.* systems A and B), but the essential condition for the reaction being progressive, the fixation of lysin to the cell components, may not have occurred; we know, at least, that the reaction is not progressive when P_0 is so small. Investigations into what would happen were the same experiments to be repeated with $P_0 = 20$, 40, etc., present insuperable technical conditions at present; *viz.*, the difficulty of separating intact red cells and ghosts from supernatant fluid. All rapid methods of separation in systems in which there is some hemolysis result in the separation of red cells plus ghosts from a supernatant fluid containing ghosts with radioactive lysin attached to them, and there seems at present to be no way in which the results obtained by such imperfect separations can be analyzed.

SUMMARY

Inhibition of hemolysis by plasma has been studied in systems containing saponin, digitonin, and sodium lauryl sulfate, using the methods developed for the study of the kinetics of progressive reactions. The results are that the progressive nature of the hemolytic reaction in saponin systems becomes less when the inhibitor is added, that the addition of inhibitor to digitonin systems has no effect on the final result although the velocity of the progressive reaction is reduced, and that the effect of plasma in lauryl sulfate systems is intermediate between the effects in saponin systems and digitonin systems. A simple explanation is that the lysin is very strongly fixed, to form an internal phase, to the cell surfaces in digitonin systems, less strongly in laurate systems, and still less strongly in saponin systems.

To answer the question as to whether, in a system in which some of the lysin forms as internal phase, the addition of an inhibitor results in a redistribution of the lysin between the internal phase and the bulk phase, sodium lauryl sulfate-S^{35} and sodium cetyl sulfate-S^{35} were prepared, and their distribution between the internal phase and the bulk phase was measured before and after the addition of plasma, the lysins being added to the cells either before or after the addition of the inhibitor. The results show that there is a large uptake of these lysins at the red cell surfaces when they are added first, and that the subsequent addition of plasma greatly reduces the quantity of lysin held in the internal phase. Further, if the inhibitor is added first and the lysin subsequently, the internal lysin phase is very incompletely formed.

Serum albumin, used in place of plasma, gives essentially similar results.

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SOME PERMEABILITY PROPERTIES OF ISOLATED RAT LIVER CELL MITOCHONDRIA*

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(Received for publication, March 16, 1956)

The technique of differential centrifugation of cell homogenates into various particulate fractions (1) has resulted in an accumulation of evidence pertaining to the importance of mitochondria in cell metabolism. The work of numerous investigators (2) has shown that certain enzymes are for the most part localized with the mitochondrial fraction of the cell. Other evidence indicates that the mitochondria are surrounded by a membrane (3, 4) which is selectively permeable (5–8). Thus it appears that the mitochondria are capable of carrying on certain specific reactions within the cell because of their selective permeability and their high concentration of certain enzymes.

In the present investigation further evidence is offered to show that the mitochondria possess differences in permeability toward certain compounds. The swelling properties of mitochondria were studied when these particulates were incubated in various media. In addition, measurements were made of the rate of penetration into mitochondria of various solutes from the media.

Methods

Mitochondria were isolated by a modification of the method of Hogeboom *et al.* (1). All work was performed at 0–4°C. except as otherwise indicated. Male rats of the Long-Evans strain, fasted for about 20 hours, were anesthetized with nembutal and the livers perfused with ice cold, modified Tyrode solution (9). The livers were forced through a stainless steel tissue sieve and the homogenates diluted with cold 0.25 M or 0.88 M sucrose solution and homogenized 5 minutes in a Potter-Elvehjem homogenizer. The liver homogenates were diluted to about 40 ml. with sucrose solution and the mitochondria isolated by differential centrifugation. All centrifugations were performed in refrigerated centrifuges. After separation of the nuclear fraction by a 10 minute centrifugation at 1300 × *g*, the mitochondria were washed three times as described previously (10) to remove the contaminating microsomes and were then used as the starting material for the penetration experiments. No attempt was made to ob-

* This investigation was performed under Contracts N7onr-29504 and Nonr-22231 between the Office of Naval Research and the University of California.

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tain the mitochondria quantitatively from a liver since it was desired to isolate the mitochondria in the shortest time possible.

The mitochondria were made up to 5 or 10 ml. with the medium to be studied, and at serial time intervals approximately 0.4 ml. aliquots of the suspension were centrifuged in specially constructed lucite tubes in a Spinco model E ultracentrifuge, care being taken to keep the temperature of the aliquot at all times as close as possible to that of the original suspension. The lucite tubes, termed "mitocrits," used for estimating the volume of the suspension, supernatant, and pellet, were $\frac{5}{8}$ inches in diameter with a bore of 3.2 mm. diameter and length of 7 cm. Fine rings were turned on the outside of the mitocrits to avoid parallax in reading the length of the respective columns with a centimeter rule marked to the 0.5 mm. The mitocrits were calibrated for the relationship between length of the column and volume content by weighing the tubes which contained various amounts of mercury. Use of these tubes enabled calculation of the sedimented pellet volume and the supernatant volume of each serial sample. The volumes of the mitochondrial pellets isolated in this manner for subsequent analysis varied from 20 to 100 microliters.

Sucrose was estimated by the method of Hassid (11) after hydrolysis with invertase, a blank being determined by omitting the invertase. Sucrose was removed from the samples by repeated extractions with boiling 80 per cent ethanol. Protein was determined by the biuret method of Kirk (12). In all cases, when reported, a sample of the original mitochondrial suspension was taken for protein analysis and the protein content of any particular serial sample calculated from this value.

Iodine^{131}-labelled bovine plasma albumin was prepared by a modification of the method of Fine and Seligman (13). The labelled albumin was dialyzed for 30 to 40 hours to remove any non-bound activity, a procedure which was carefully checked by showing that less than 10^{-3} per cent of the activity remaining after 30 to 40 hours was dialyzable.

Sodium chloride solutions containing sodium^{24} were prepared by adding an excess of hydrochloric acid to sodium carbonate containing sodium^{24} and evaporating this solution to dryness. The residue was then diluted to the desired concentration with water. Radioactive potassium chloride solutions were prepared from a mixture of potassium^{42} and ^{43}. The potassium$^{42\text{-}43}$, manufactured by cyclotron bombardment, was evaporated to dryness and diluted with potassium chloride solution. All radioactivity measurements were made with a scintilation counter.

As an approximation the equation of Collander and Bärlund (14) was used to describe the penetration of solute into the mitochondria. This equation assumes constant cell volume and cell surface area, conditions not realized in the present investigation. However, because the penetration appears to follow Fick's law of diffusion, the equation may be utilized for purposes of comparison of half-times for penetration of a solute. This equation may be written as: $C_t = C\infty(1 - e^{-Kt})$ in which C_t is the mitochondrial pellet concentration at time t and $C\infty$ is the mitochondrial pellet concentration at equilibrium and K includes the parameters: diffusion coefficient, mitochondrial surface area, mitochondrial volume, and mitochondrial membrane thickness. Half-times for change in mitochondrial pellet concentration were determined from the relationship: $t_{1/2} = \frac{\ln 2}{K}$, in which K was determined by plotting $\ln (C\infty - C_t)$ against time, the slope being equal to K.

RESULTS

Estimation of Trapped Fluid in Mitochondrial Pellets

Preliminary experiments were performed on mitochondria isolated in 0.88 M sucrose. Analysis of these isolated mitochondrial pellets showed that they

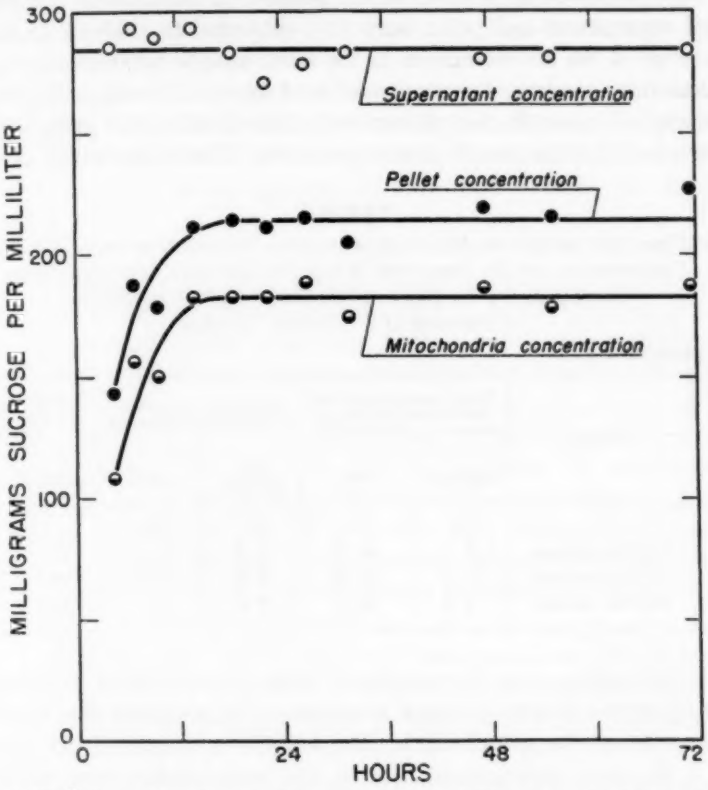

FIG. 1. The effect of incubation of mitochondria in 0.88 M sucrose solution at 0–4°C. with respect to whole pellet sucrose concentration and mitochondrial sucrose concentration, the latter based on non-permeation of I^{131}-labelled albumin.

contained a high concentration of sucrose, which was indicative of penetration of sucrose into the mitochondria. However, because the interstitial space of the pellet was unknown it could not be definitely concluded that this sucrose was not entirely located in the fluid between the mitochondria. I^{131}-labelled bovine plasma albumin was chosen as a molecule possibly too large to enter the mitochondria, which if true would allow the determination of the interstitial space of the pellet.

Mitochondria were isolated in two experiments in 0.25 M sucrose and in the

third in 0.88 M sucrose. The mitochondria were kept in the same media at 0–4°C. but with the addition of I^{131}-labelled albumin at a concentration of 2 to 13 mg. of albumin per ml. At successive time intervals an aliquot was centrifuged for 15 minutes at 24,600 × *g* in mitocrit tubes. The supernatant was removed and the top of the pellet and sides of the tubes rinsed three times with water, followed by removal of the pellet with three rinses of water. The isolated supernatant and pellet were then subjected to analysis. In all cases the average of the concentrations of the serial sample supernatants was used for calculations involving the concentration of solute in the suspending medium. The change of concentration of sucrose in the mitochondrial pellet with time is shown in Fig. 1 for one of these experiments. The concentration of sucrose

TABLE I

Half-Times for Increase in Mitochondrial Sucrose Concentration and Pellet Sucrose Concentration, and the Range over Which the Calculated Interstitial Space Changed during the Course of the Experiments, Assuming no Penetration of I^{131}-Labeled Albumin

Temperature 0–4°C.

Rat No.	Medium	Time measurements were made after addition of sucrose to mitochondria		Half-time for change in sucrose concentration		Interstitial space as per cent of pellet volume	
		Beginning	End	Mitochondria	Pellet	Beginning	End
		hrs.	*hrs.*	*hrs.*	*hrs.*		
1	0.25 M sucrose	3.7	60.3	9.2	8.2	17	33
2	0.25 M sucrose	3.2	71.6	4.4	4.6	16	30
3	0.88 M sucrose	3.2	55.5	2.9	3.1	21	41

in the mitochondria alone was calculated from the total pellet concentration, correcting for the interstitial space as estimated by assuming that no labelled albumin entered the mitochondria. The half-times calculated are shown in Table I. In these experiments, because the mitochondria were maintained at the same temperature throughout the experiment, time zero was taken as the time at which sucrose was first added to the liver pulp.

It may be seen that the mitochondria are permeable to sucrose even at temperatures in the range of 0–4°C. The variation in half-times among separate experiments was much greater than the difference between half-times for changes in the whole pellet sucrose concentration and mitochondrial sucrose concentration, which agreed fairly well. For this reason it was concluded that determination of the half-time for changes in the whole pellet concentration could be used as an index for the changes in concentration occurring in the mitochondria.

Table I also shows the range over which the calculated interstitial spaces

changed during the period of the experiments. There was a continual increase in the radioactivity of the pellet with time, which was probably due either to a slow penetration of the mitochondria by albumin or to adsorption of the labelled albumin onto the mitochondria. The minimum interstitial space calculated in these experiments was in the neighborhood of 18 per cent. This 18 per cent value is probably close to the true interstitial space because such values are recorded at a time near zero and minimize the effect of any penetration or adsorption of albumin.

The foregoing conclusion is based on the assumption that adsorption by, or penetration into, the mitochondria by labelled albumin is small as compared to the amount of the label in solution in the interstitial space. The next experiments dealt with an attempt to determine whether the assumption was correct.

An experiment was carried out to see how much of the activity in the pellet could be removed by repeated washing. Mitochondria were isolated in 0.25 M sucrose, and 47 mg. of albumin labelled with 9 μc. of I^{131} added to the suspension, which had a final total volume of 10 ml. The mitochondria were allowed to stand in the albumin-sucrose medium for 1 hour at 0–4°C. After 1 hour 2 ml. duplicate samples of the mitochondrial suspension were transferred to centrifuge tubes and centrifuged for 15 minutes at 24,000 × *g*. 1 ml. of supernatant was removed from each for counting, the remainder being discarded. The top of the pellet and inside of the tube were rinsed three times with 0.25 M sucrose. 2 ml. of 0.25 M sucrose were added and the pellet was resuspended and recentrifuged. This procedure was repeated two more times at approximately 50 minute intervals, taking samples of the supernatant each time. At the end of the last sedimentation, the pellet was removed for radioactivity assay, together with an aliquot of the supernatant. Calculations based on a separate mitocrit determination from the same original sample showed that the pellet was diluted by a factor of ten at each wash. The radioactivity concentration in the pellet was calculated by adding to the activity of the final washed pellet the total activity removed at each wash. Fig. 2 shows the results of such washing on the removal of the labelled albumin. It may be seen that three washes of the mitochondria reduced the activity concentration of the pellet to 15 per cent of the original value, indicating that the bulk of the activity was readily removable.

Another experiment was devised in which the rate of liberation of the tightly bound labelled albumin could be studied. Mitochondria were isolated in 0.25 M sucrose and incubated for 1.5 hours at 0–4°C. in 0.25 M sucrose containing 9 μc. of I^{131}-labelled albumin. Following incubation the pellet was twice washed with 0.25 M sucrose by means of 8 minute centrifugations at 27,950 × *g*, which by dilution would have reduced the free albumin to less than 2 per cent of the original value. The pellet was resuspended in 0.25 M sucrose and allowed to

remain at 0–4°C. At serial time intervals, samples were taken and the concentration of radioactivity determined for the supernatant and pellet. As may be seen from Fig. 3, the activity was seen to leave the mitochondria slowly,

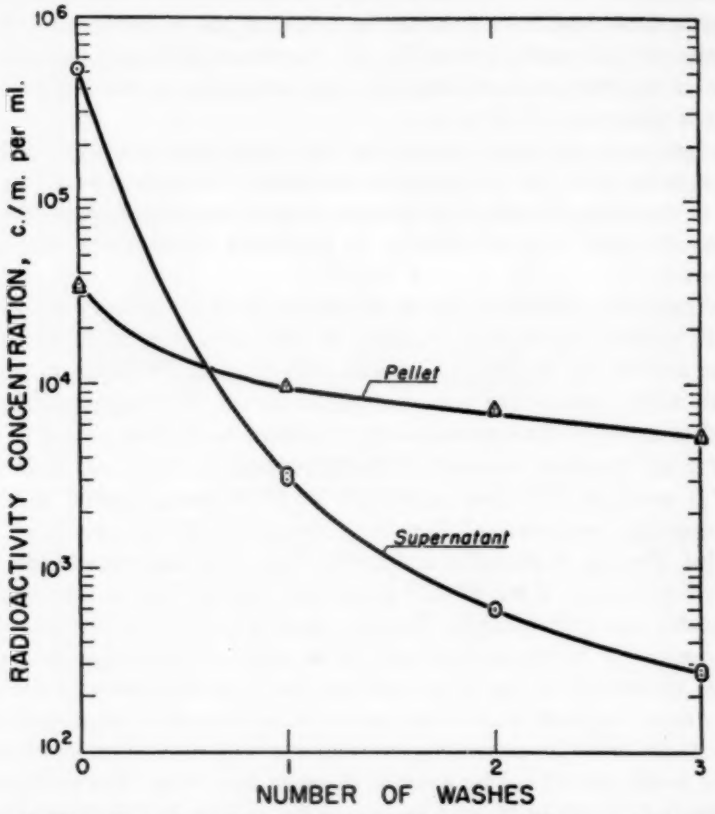

FIG. 2. The effect of washing a mitochondrial pellet with respect to removal of I^{131}-labelled albumin. At each wash the pellet was diluted by approximately a factor of 10 with fresh 0.25 M sucrose.

and at 19 hours the pellet activity concentration was still 16 times higher than that of the supernatant.

Because in the washing experiment the pellet activity concentration was reduced to about 30 per cent of the original by the first wash, which on the basis of dilution alone, should have reduced it to about 10 per cent, it can be shown by a simultaneous equation that only 22 per cent of the label in the pellet was originally associated with the mitochondria. This 22 per cent probably represents adsorption by or penetration into the mitochondria and is removed very slowly as indicated in Fig. 3.

The important consideration is that the bulk of the labelled albumin in the pellet, *i.e.* about 80 per cent, described in the previous sucrose penetration experiments represents interstitial space, and that a valid estimate of the space may be made. Furthermore, because the interstitial space as measured

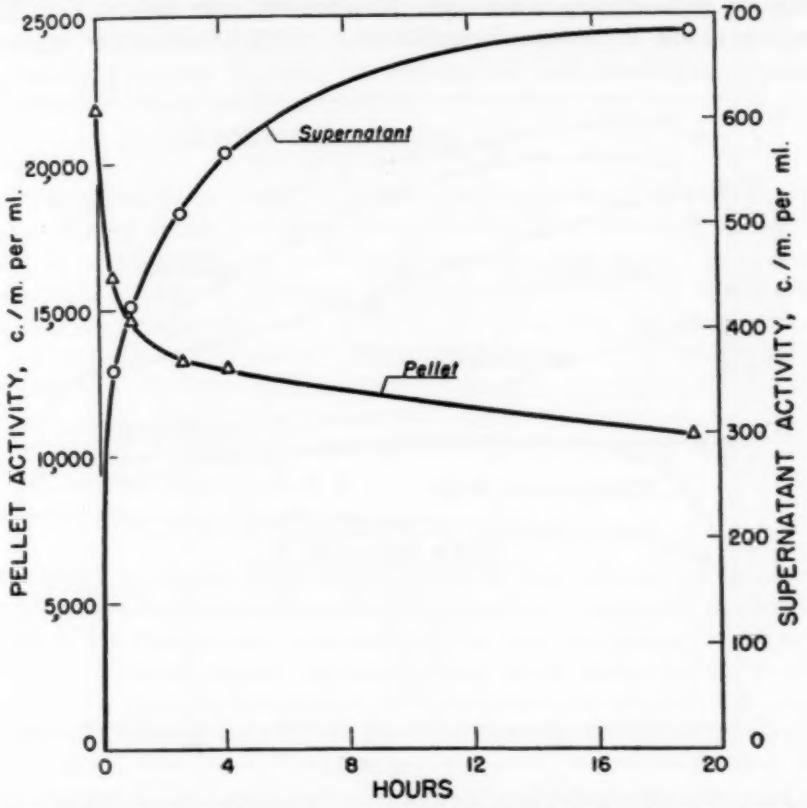

FIG. 3. The rate at which the concentration of I^{131}-labelled albumin was reduced in a mitochondrial pellet and the rate it appeared in the suspending medium when a pellet containing labelled albumin was resuspended in 0.25 M sucrose solution.

is small, *i.e.* less than 20 per cent, and because the calculated half-times for penetration of sucrose into the mitochondria and whole pellet are approximately the same, it would appear valid to use the concentration changes of various solutes occurring in the whole pellet as an index to the concentration changes of those solutes occurring within the mitochondria.

Penetration Experiments

In the following experiments a modification of the original scheme of centrifugation was used to allow a more rapid isolation of mitochondria. After

isolation of the nuclear fraction as described previously, the mitochondria were sedimented by an 8 minute centrifugation at 17,590 × *g*. Washing of the mitochondria was carried out by several resuspensions of the pellets with fresh sucrose solution in 8 mm. bore tubes and recentrifugation as above to remove contaminating microsomes. Mitochondria were isolated in 0.25 M sucrose except in the case of experiments in which 0.88 M sucrose was used.

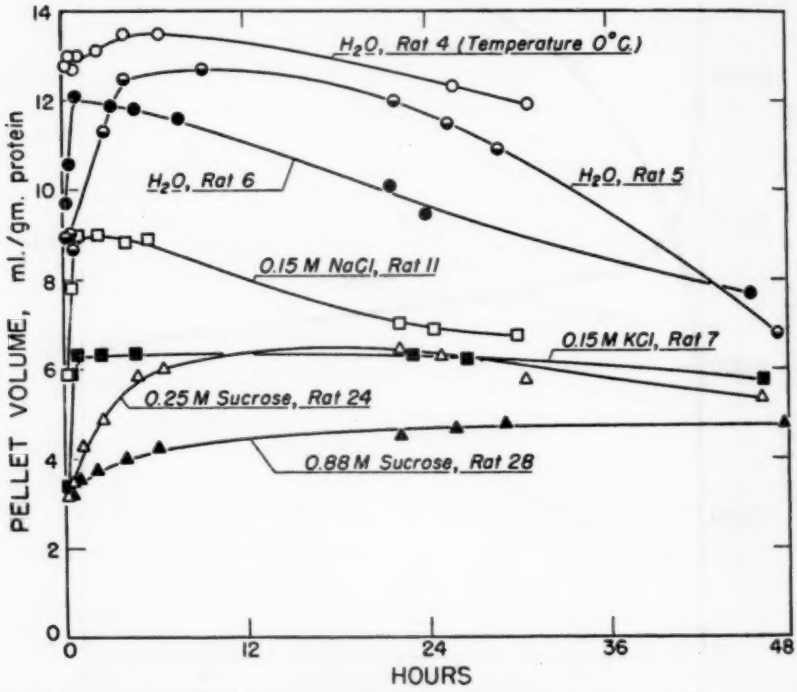

FIG. 4. The effect of distilled water and various solutions on the swelling properties of mitochondria at 12.5°C.

In all cases the isolated mitochondria were made to a volume of 5 ml. in the medium under study. At successive time intervals an aliquot was centrifuged for 8 minutes at 31,000 × *g* in mitocrit tubes and the pellets and supernatants analyzed as described previously. In all these experiments time zero was taken as the time at which the mitochondria were placed in the medium under study; *i.e.*, not including the time for isolation. This was done because the experiments were carried out at a temperature different from that used in isolation and in some instances mitochondria were placed in solutions differing from that of the isolation medium. The procedure allowed isolation of mitochondria from the liver pulp in 1.7 hours.

In experiments in which compounds purposely were not added to alter the pH of the resulting 5 ml. suspension, the pH was approximately 7.4. This was determined by preliminary experiments which showed that in each case in which mitochondria were isolated in sucrose and finally suspended in sucrose, KCl, or NaCl, the pH of the resulting suspension, as measured by the glass electrode, was between pH 7.3 and 7.5. When I^{131}-labelled albumin was added, the resulting pH was 7.1. Each pH measurement was obtained using mitochondria from a separate whole rat liver.

The Effect of Distilled Water

Mitochondria when placed in distilled water were observed to swell rapidly before the first measurement could be made (within 10 minutes) followed by a slower rate of swelling. In water the mitochondria were found to swell more rapidly and to larger volumes than in any other medium studied.[1] The results or these experiments are shown in Fig. 4. For comparison, the effect of other media on mitochondrial volume changes is also shown. Mitochondrial pellet volumes are expressed as milliliters of pellet per gram of mitochondrial protein present at zero time. It may be seen that neither the degree nor the rate of initial swelling was reduced by lowering the temperature from 12.5 to 0°C. The results are summarized in Table II.

The Effect of Potassium Chloride Solutions at 12.5°C.

In these experiments the amount of potassium which entered the mitochondria was determined by adding radioactive potassium to the potassium chloride medium. The concentration of potassium in the mitochondrial pellet was then calculated from the volume and radioactivity of the pellet and the specific activity of the suspending medium. This introduces the possible error of exchange of potassium from the medium with bound potassium already in the mitochondria. The term "bound" as used here refers to compounds which are not removed by the repeated washing of the mitochondria during the isolation procedure. The exchange would result in a value higher than the true concentration of free diffusible potassium in the pellet entering from the suspension medium.

The error, if it occurred, would be small because the bound potassium of isolated mitochondria is only 16 to 17 per cent of the maximum content found to be reached by the granules after incubation in 0.15 M KCl, this value being 31 mg. per gm. of mitochondrial protein. Griswold and Pace (16) have reported a bound potassium content of 5.3 mg. of potassium per gm. of mitochondrial protein when the mitochondria were isolated by the same method used here. This agrees with the value reported by Stanbury and Mudge (17) of 4.9 mg.

[1] An exception was seen after prolonged treatment in the medium of MacFarlane and Spencer (15).

TABLE II

Summary Table of Penetration Experiments

Mitochondria were isolated by differential centrifugation at 0–4°C. in 0.25 M sucrose except in the case of 0.88 M sucrose penetration experiments in which case the particles were isolated in 0.88 M sucrose. The time for isolation of mitochondria varied between 1.6 and 1.8 hours. Time intervals do not include isolation time. For further details see text.

Rat No.	Incubation medium	Solute measured	Incubation temperature	Incubation medium	Extrapolated zero time pellet concentration (as per cent of equilibrium)	Pellet volume (ml./gm. protein)		Time initial and maximum pellet volumes measured		Maximum concentration of solute reached in pellet (per cent of supernatant concentration)	Time maximum solute concentration reached in pellet	Half-time for penetration of solute
						Initial	Maximum	Initial	Maximum			
			°C.	*p*H				*hrs.*	*hrs.*		*hrs.*	*hrs.*
4	H_2O	—	0	7.4	—	12.8	13.5	0.1	4	—	—	—
5	H_2O	—	12.5	7.4	—	9.0	12.7	0.1	4	—	—	—
6	H_2O	—	12.5	7.4	—	9.5	12.1	0.1	0.9	—	—	—
7	0.15 M KCl	K	12.5	7.4	55	3.4	6.3	0.1	8	94	1.2	0.1
8	0.15 M KCl	K	12.5	7.4	—	3.7	6.7	0.1	8	92	0.6	<0.1
9	Trace K^{42} and K^{43} in 0.15 M NaCl	K	12.5	7.4	—	4.0	7.6	0.2	5	538	46	<0.1
10	0.15 M NaCl	Na	12.5	7.4	36	3.4	5.0	0.2	1	94	1	0.4
11	0.15 M NaCl	Na	12.5	7.4	—	5.9	9.0	0.1	1	96	0.4	<0.1
12	0.15 M NaCl–Na_2CO_3	Na	12.5	8	48	3.5	8.2	0.2	8	99	6	1.2
13	0.15 M NaCl–Na_2CO_3	Na	12.5	8	63	4.0	8.4	0.2	7	99	6	1.1
14	0.15 M NaCl–Na_2CO_3	Na	12.5	8	53	3.6	7.3	0.2	7	100	8	1.2
15	0.15 M NaCl–Na_2CO_3– KCl; Na/K = 1/5	Na	12.5	8	74	3.9	8.4	0.1	4	95	4	0.4
16	0.15 M NaCl–Na_2CO_3– KCl; Na/K = 1/29	Na	12.5	8	73	3.5	9.0	0.1	4	97	5	0.8
17	0.073 M NaCl–Na_2CO_3	Na	12.5	8	55	3.9	7.7	0.1	7	102	4	0.9
18	0.06 M Na*	Na	12.5	7.4	—	4.6	16.1	0.1	44	101	—	—
							12.0		23	96		
							6.0		6	97		

19	0.06 M Na*	Na	28.0	7.4	—	4.6	6.0	0.2	1	99	—	—
20	I^{131} albumin 4.7 mg./ml. in 0.15 M NaCl	Albumin	12.5	7.1	—	6.7	10.0	0.1	1	27	—	—
21	I^{131} albumin 4.2 mg./ml. in 0.15 M NaCl	Albumin	12.5	7.1	—	4.1	7.8	0.1	1	21	—	—
22	0.25 M sucrose	Sucrose	12.5	7.4	50	3.7	5.3	0.1	14	76	16	3.1
23	0.25 M sucrose	Sucrose	12.5	7.4	78	3.9	6.0	0.1	9	76	10	1.7
24	0.25 M sucrose	Sucrose	12.5	7.4	64	3.2	6.4	0.1	14	86	11	1.9
25	0.25 M sucrose	Sucrose	12.5	7.4	85	3.5	7.6	0.2	6	94	5	0.9
26	0.88 M sucrose	Sucrose	12.5	7.4	78	3.0	4.2	0.1	13	88	12	1.9
27	0.88 M sucrose	Sucrose	12.5	7.4	82	2.6	4.8	0.1	12	90	13	2.7
28	0.88 M sucrose	Sucrose	12.5	7.4	75	3.2	4.8	0.1	29	88	22	4.7

* Medium of MacFarlane and Spencer (15).

of potassium per gm. of mitochondrial protein (based on 16 per cent nitrogen for mitochondrial protein). Furthermore, the latter authors have shown that under conditions similar to those used here there is negligible exchange between bound mitochondrial potassium and the potassium of the suspending medium.

When mitochondria were incubated with 0.15 M KCl at 12.5°C., the mitochondria swelled rapidly and the concentration of diffusible potassium in the pellet increased rapidly from zero to 93 per cent of the supernatant concentration. This is shown in Fig. 5. In two experiments the half-times for penetration of potassium were calculated to be approximately 0.1 hour. The decrease in pellet volume seen after 24 hours is probably due to rupture of some of the mitochondrial membranes.

Incubation of mitochondria at 12.5°C. in 0.15 M NaCl containing a trace amount of carrier-free radioactive potassium resulted in the pellet activity concentration increasing rapidly to a maximum of 98 per cent of the medium activity concentration and remaining at this level for 6 hours. Following this time the activity concentration of the pellet rose slowly to a value of 538 per cent of the medium concentration at 46 hours. Thus it may be concluded that exchange with the bound potassium does not occur to any appreciable extent during the first 6 hours. Consequently, analysis for diffusible potassium entering the mitochondrial pellet as used here is considered valid. The data are summarized in Table II.

The Effect of Sodium Chloride Solutions at 12.5°C.

In these experiments calculations for the sodium entering the mitochondrial pellet from the suspension medium again were based on radioactivity. Mitochondria were incubated in sodium chloride solutions containing sodium^{24}. The concentration of sodium in the mitochondria was calculated from the volume and radioactivity of the pellet and the specific activity of the supernatant.

The maximum sodium uptake by the mitochondria from 0.15 M NaCl solutions at pH 7.4 was 14 and 27 mg. of sodium per gm. of mitochondrial protein. Spector (18) has reported between 0.5 and 1.7 mg. of sodium per gm. of protein (based on mitochondrial protein containing 16 per cent nitrogen) and Griswold and Pace (16) have found 0.35 mg. of sodium per gm. of protein for the bound mitochondrial sodium. Therefore exchange, if it occurred, would represent only 1 to 12 per cent of the total sodium found by this method.

In all the experiments with 0.15 M NaCl at various hydrogen ion concentrations the maximum concentration reached in the pellet was between 94 and 102 per cent of the supernatant concentration. When the concentration of sodium was made 0.0048, 0.024, and 0.073 M with the total made to 0.15 M in the first two by the addition of potassium chloride, the maximum concen-

trations of sodium reached by the pellets were still only 95, 97, and 102 per cent of the supernatant concentration respectively. In the case of 0.0048 M sodium the maximum uptake of sodium by the pellet was 0.85 mg. per gm. of mitochondrial protein, which is about equal to the amount of bound mitochondrial sodium. This indicates a very low exchange, if any, of the bound

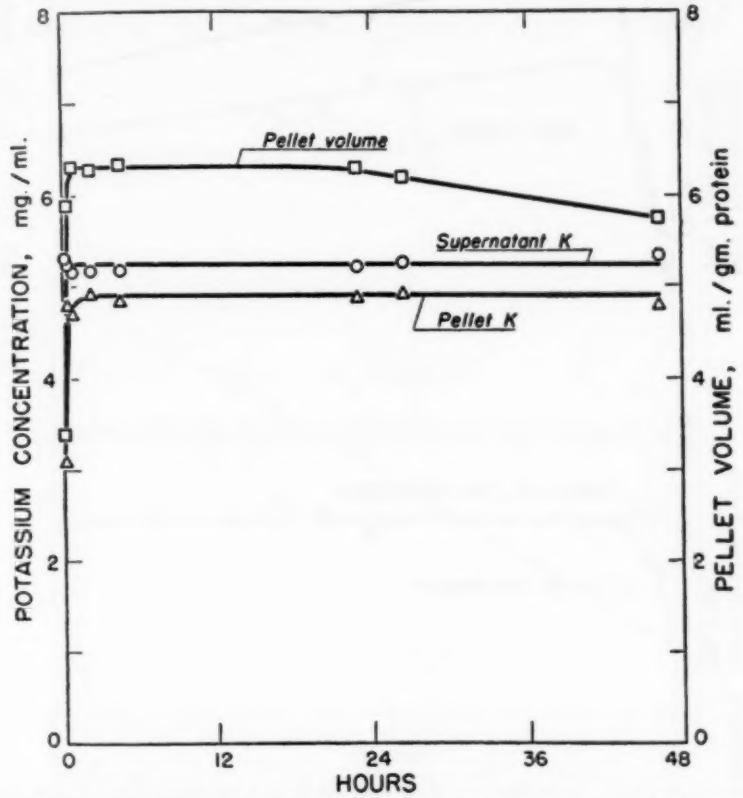

FIG. 5. Potassium uptake by mitochondria suspended in 0.15 M potassium chloride. Temperature, 12.5°C; pH, 7.4.

sodium with sodium taken up from the suspending medium since at all concentrations of sodium the equilibrium concentration in the pellet percentagewise was about the same. The conclusion is based on the fact that a mitochondrial pellet is about 90 per cent water (15) and on the evidence that the sodium label rapidly enters this water space and comes to equilibrium with the suspending medium sodium concentration. If appreciable exchange occurred, the calculated sodium concentration of the pellet would be based on the label in the water space as well as the bound sodium and would result in a

calculated pellet sodium concentration that is considerably higher than the suspending medium.

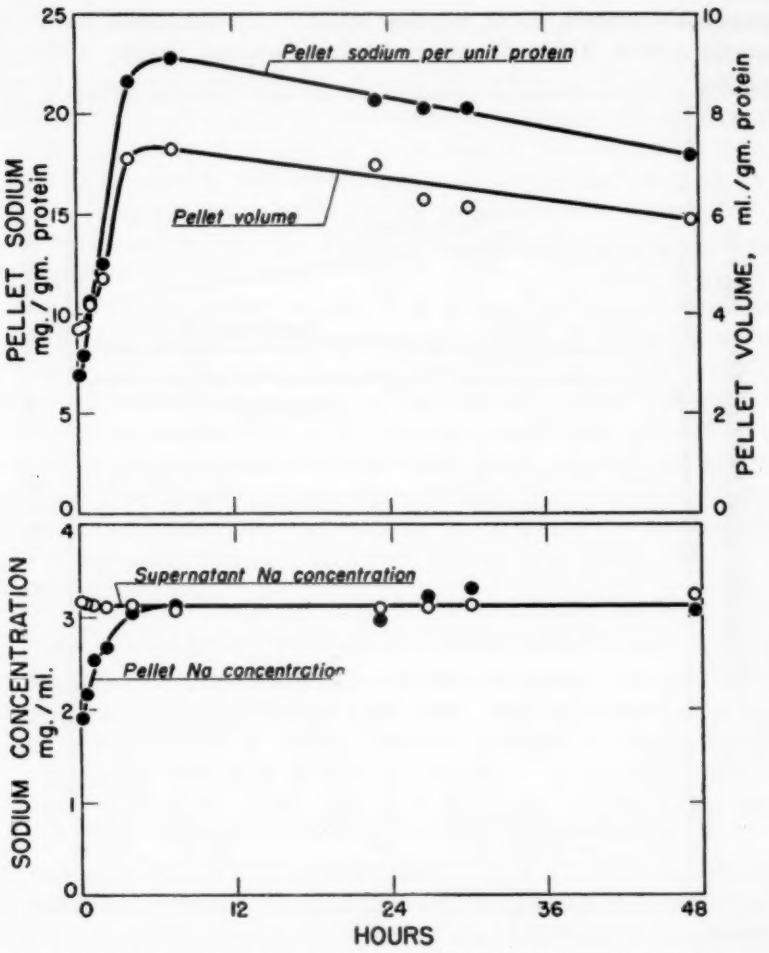

FIG. 6. Sodium uptake by mitochondria suspended in 0.15 M sodium chloride. Temperature, 12.5°C; pH, 8.0. Curve indicates a slowing of sodium penetration due to change in pH. At pH 7.4 curves for sodium are similar to those for potassium shown in Fig. 5.

The half-times for penetration when mitochondria were incubated in 0.15 M NaCl at 12.5°C. are shown in Table II. It may be seen that at pH 7.4 sodium enters at about the same rate as does potassium. When the pH was altered by the presence of sodium carbonate, the rate of penetration of sodium was

changed. The data show that alteration of the pH from about 7.4 to 8.0 caused a decreased rate of penetration. This is in agreement with the work of Cleland (7) and Raaflaub (8) based on changes in the rate of swelling of mitochondria as measured by photometric means. Fig. 6 shows an example of the changes

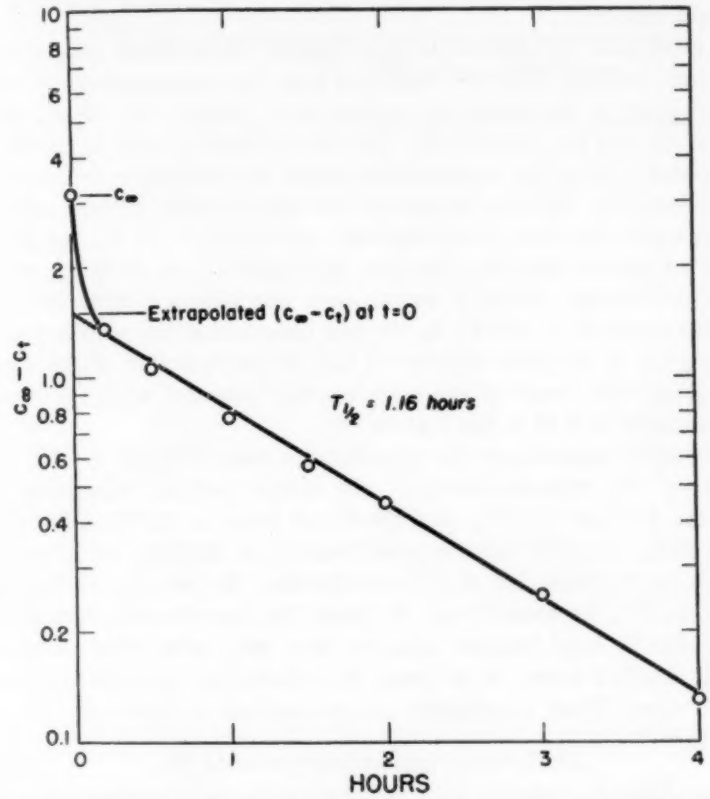

FIG. 7. Graphical determination of the half-time for penetration of sodium as determined by plotting the smooth curve of change in pellet sodium concentration with time shown in Fig. 6.

in pellet sodium concentration when mitochondria were incubated in 0.15 M sodium chloride–sodium carbonate solution at pH 8.0. Fig. 7 shows the graphical analysis for the half-time of penetration of sodium ion in this experiment.

MacFarlane and Spencer (15) have reported that mitochondria are able to concentrate sodium and potassium against an external concentration gradient. In their experiments mitochondria were incubated at 28°C. in a medium composed of: 0.05 M KCl, 0.02 M sodium L-glutamate, 0.005 M $MgSO_4$, 0.01 M Na_2HPO_4–KH_2PO_4 (pH 7.4), 0.00003 M cytochrome *c*, and 0.0015 M adenosine-

5-monophosphate. The medium contained sodium and potassium at concencentrations of 0.81 mg. per ml. and 2.07 mg. per ml. respectively. These investigators found an increase in both sodium and potassium over that of the suspension medium when mitochondria were incubated for 10, 30, 60, and 90 minutes with AMP present in the medium. No increase was found when AMP was omitted.

The experiment of MacFarlane and Spencer was repeated using the same incubation medium, with the exception that the concentrations of sodium and potassium in the incubation medium were changed to 1.38 mg. per ml. and 1.60 mg. per ml. respectively. The concentration of sodium entering the mitochondrial pellet was measured by means of radioactive sodium as previously described. By this method no increase in pellet sodium concentration was found over that of the suspension medium at 9, 28, 64, and 146 minutes or for various samples after this time taken up to 25 hours after the start of incubation. Negative results were also obtained when the experiment was repeated at 12.5°C. In the two experiments the measured sodium concentration in the pellet reached 97 and 99 per cent that of the suspending medium. This result is the same as that observed when mitochondria were incubated in 0.15 M NaCl at 12.5°C.

In the above experiments the mitochondria were observed to swell to approximately the volumes observed with simple sodium chloride solutions during the 1st hour at 28°C. and the first 6 hours at 12.5°C. After the 1st hour at 28°C. the pellet volumes were observed to decrease, which was probably due to disintegration of the mitochondria. In the case of the experiment at 12.5°C., between 20 and 30 hours, the mitochondria were found to have swollen to large volumes equal to those seen when mitochondria were placed in distilled water. At 44 hours the volume had exceeded that seen in distilled water. These experiments are summarized in Table II.

The Effect of Sucrose Solutions at 12.5°C.

These studies are different from those described in the preliminary experiments in that no albumin was present and the incubation temperature was 12.5°C. instead of 0–4°C. The effects of sucrose solutions may, therefore, be compared with the previously described effects of sodium chloride and potassium chloride solutions.

Experiments performed in 0.25 M sucrose at pH 7.4 showed that sucrose entered the mitochondria more slowly than sodium and potassium at the same pH. The half-times for sucrose penetration in four experiments were determined to be: 3.1, 1.7, 1.9, and 0.9 hours. Fig. 8 shows the change in sucrose concentration in the mitochondrial pellet with time for one of these experiments, and Fig. 9 shows the graphical determination of the half time for penetration of sucrose in the same experiment. The maximum sucrose

concentration reached in the pellet as a per cent of the supernatant concentration was 76, 76, 86 and 94 per cent respectively. The times at which the maximum pellet concentrations were reached were: 16, 10, 11, and 5 hours respectively. The maximal mitochondrial volumes attained were similar to

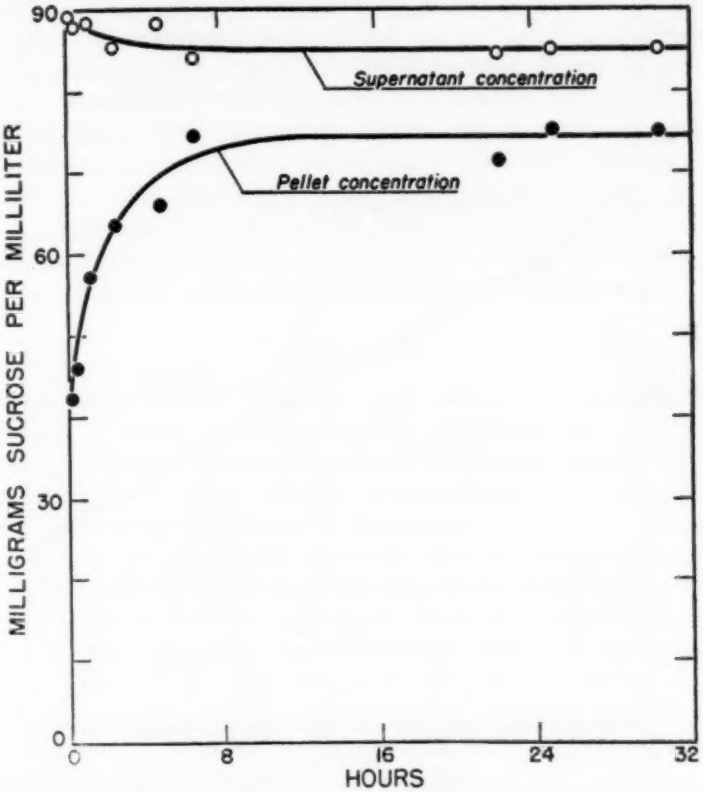

FIG. 8. Sucrose uptake by mitochondria suspended in 0.25 M sucrose solution. Temperature, 12.5°C.; pH, 7.4.

those observed when mitochondria were incubated in potassium or sodium chloride solutions.

When mitochondria were incubated in 0.88 M sucrose, the half-time for penetration of sucrose did not differ greatly from that in 0.25 M sucrose. In 0.88 M sucrose the half-times for penetration of sucrose were: 1.9, 2.7, and 4.7 hours and the sucrose concentrations reached in the pellets as a per cent of the supernatant concentrations were: 88, 90, and 88 per cent respectively. The times at which the maximum pellet sucrose concentrations were reached were: 12, 13, and 22 hours respectively.

The maximum volume attained by mitochondria in 0.88 M sucrose was somewhat less than that observed with 0.25 M sucrose. Data for both 0.25 and 0.88 M sucrose experiments are summarized in Table II.

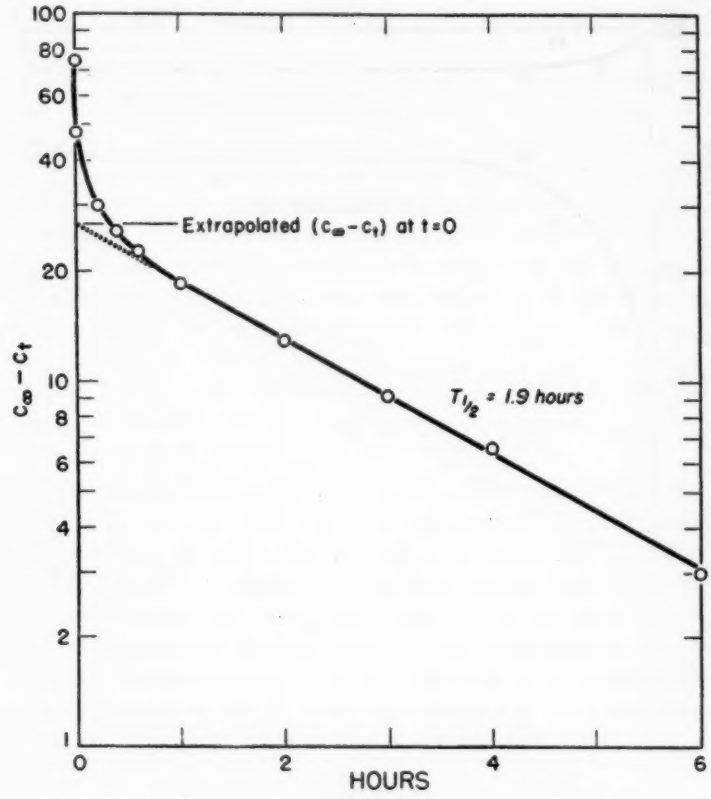

FIG. 9. Graphical determination of half-time for penetration of sucrose as determined by plotting the smooth curve of change in sucrose concentration with time shown in Fig. 8.

The Effect of I^{131}-Labelled Albumin in 0.15 M Sodium Chloride at 12.5°C.

Two experiments were performed in which mitochondria were incubated at 12.5°C. and at an approximate pH of 7.1 in 0.15 M NaCl containing 4.7 and 4.2 mg. per ml. of I^{131}-labelled albumin. These experiments differ from the preliminary experiments with albumin in that the temperature was different and the medium contained sodium chloride, the cation of which penetrates mitochondria very rapidly as compared to sucrose. The amount of albumin in the pellet was determined by the radioactivity of the pellet in the same manner as in the sodium and potassium experiments. There was a

slow increase in albumin concentration in the pellet which reached only 21 and 27 per cent of the supernatant concentration after 46 hours' incubation from an initial concentration of 17 and 18 per cent respectively. These data are shown in Table II.

To rule out the possibility that this extremely slow penetration as compared to previous experiments with sucrose, sodium, and potassium was due to the change in pH from 7.4 to 7.1, the experiment described below was carried out. Mitochondria were incubated in a solution buffered to pH 7.1 by use of Michaelis' "universal buffer" (19) which is isotonic with blood and contained sodium at a concentration of 0.15 M. Under these conditions sodium ion entered the mitochondria with a half-time of 0.3 hour which is within the range of the half-times for sodium penetration determined for 0.15 M NaCl solutions at pH 7.4. Thus it may be concluded that if albumin enters at all, this compound penetrates isolated mitochondria extremely slowly.

DISCUSSION

The experiments reported here show that isolated rat liver mitochondria are permeable to water, sodium, potassium, and sucrose and are relatively impermeable to albumin. The more rapid swelling of mitochondria in distilled water as compared to sodium chloride or potassium chloride solutions indicates that water enters very rapidly in comparison to the rate at which these solutes enter. At pH 7.4 sucrose enters the mitochondria more slowly than sodium or potassium and albumin enters very slowly, if at all. The fact that the ratio of pellet albumin concentration to supernatant albumin concentration after 46 hours' incubation was considerably lower than the ratio of the equilibrium pellet sucrose concentration to supernatant sucrose concentration, and that most of the pellet albumin can be considered to be in the interstitial space of the pellet, clearly shows that isolated rat liver mitochondria are more permeable to sucrose than to albumin.

The findings indicate that some type of barrier to free diffusion exists. Further evidence for this is seen when one considers the free diffusion of sucrose into a sphere of water the size of a mitochondrion as compared to the measured rate of penetration of sucrose into mitochondria. Fig. 8 shows the data obtained for change in mitochondrial pellet sucrose concentration with time for rat 24 at 12.5°C. in 0.25 M sucrose. Fig. 9 shows the smooth curve from Fig. 8 plotted as ln $(C\infty - C_t)$ against time, and from the slope of the straight line portion a value of 1.9 hours was obtained for the half-time of increase in pellet sucrose concentration.

The following equation for free diffusion of a solute into a sphere is given by Jacobs (20):

$$\pi/c = 1 - \frac{6}{\pi^2}\left(e^{-\pi^2 Dt/r^2} + \tfrac{1}{4}e^{-4\pi^2 Dt/r^2} + \cdots\right)$$

in which $\bar{q}$ is the average concentration of solute in the sphere at time t, and c is the constant external concentration of solute. The value r is the radius of the sphere and D is the diffusion coefficient. By estimating a mean value for the radius of a mitochondrion, one can calculate the relationship between concentration of solute in the mitochondrion and time, assuming that the membrane offers no resistance to free diffusion and the interior is water. The diffusion coefficient for sucrose in aqueous solution at a concentration of 1 gm.-mole per liter and at a temperature of 12°C. is 3.0×10^{-6} cm.^{2} per second (21). For a sphere of water of 1 micron radius immersed in 1 M sucrose, the half-time for increase in average sucrose concentration inside the sphere may be calculated to be 0.24 millisecond. Similar calculations show that even for a 10 micron radius the half-time is only 24 milliseconds. Because the radii of the mitochondria used in the present experiments were certainly less than 10 microns, there is clearly a very slow penetration of sucrose into the mitochondria as compared to free diffusion of sucrose into a sphere of water of the same size range.

The equation of Jacobs does not assume instantaneous mixing of solute within the sphere as does the equation of Collander and Bärlund, given earlier. It may be seen, however, that owing to the rapid rate at which equilibrium is reached for spheres in the size range of cells, for all practical purposes instantaneous mixing can be assumed to occur within the mitochondrion, provided the intramitochondrial contents do not restrict free aqueous diffusion to any great extent.

A deviation from a straight line like that shown in Figs. 7 and 9 at a time near zero was observed in all experiments and would not be seen if the equation of Collander and Bärlund were followed experimentally. The magnitude of this deviation at time zero is indicated in Table II under the column "extrapolated zero time pellet concentration." The values were calculated as follows: The extrapolated zero time pellet concentration is $C\infty - C\infty_e$ in which $C\infty$ is the experimentally determined pellet solute concentration at equilibrium and $C\infty_e$ is the value obtained by extrapolating the straight line portion of the logarithmic plot to zero time. The extrapolated zero time pellet value is expressed as a per cent by the relation $\frac{C\infty - C\infty_e}{C\infty} \times 100$ and represents the concentration of solute reached in the pellet by one or more rate constants differing from the rate constant reported. From these values it would seem that a large portion of the solute penetrated the mitochondria by a more rapid process than is indicated by the calculated rate constants. In the case of sucrose this is partially explained by sucrose that entered during the isolation procedure, because zero time was actually taken as 1.7 hours after the introduction of sucrose, when a temperature change was made.

Furthermore, in use of the equation of Collander and Bärlund for a description of the penetration process of mitochondrial pellets by solutes one would expect a deviation at a time near zero because mitochondria swell during the penetration process. The rate constant determined by this equation is equal to: $\frac{AD}{Vx}$, in which A is the particle surface area available for free diffusion, V the particle volume, x the membrane thickness, and D the diffusion coefficient. The relationship between the ratio of surface area to volume, A/V, and volume, V, for a sphere may be determined from the equations for surface area and volume which are: $A = 4\pi r^2$ and $V = 4/3\pi r^3$ respectively. Solving for the relationship between A/V and V by eliminating r results in the equation: $(A/V)^3 = \frac{36\pi}{V}$. Because the ratio of surface area to volume of a sphere decreases as the volume increases according to $(A/V)^3 = \frac{36\pi}{V}$, there will be a decrease in the rate constant as the volume increases, assuming that no changes occur in the values of x or D. For a doubling in particle volume, which is the approximate mitochondrial pellet volume change between the first measurement and the maximum volume reached, the rate constant is 26 per cent greater at the original volume as compared to the time at which the volume is doubled. Consequently, the rate constant as used here is not constant but decreases as the mitochondria swell.

It should be pointed out that the rate constant is independent of a constant interstitial space. This is shown by the following consideration. The analysis for K is made by rewriting the equation of Collander and Bärlund as:

$$\ln C\infty - \ln (C\infty - C_t) = Kt$$

The term $\ln C\infty$ may be dropped because it is a constant and does not alter the slope of the curve obtained by plotting $\ln(C\infty - C_t)$against time, as shown by the following. The slope K equals:

$$\frac{[\ln C\infty - \ln(C\infty - C_{t_2})] - [\ln C\infty - \ln(C\infty - C_{t_1})]}{t_2 - t_1}$$

which may be simplified to:

$$\frac{\ln (C\infty - C_{t_1}) - \ln(C\infty - C_{t_2})}{t_2 - t_1}$$

and the determination of the slope K by this method is independent of the first constant logarithmic term, $\ln C\infty$. The total equation used is then:

$$\ln\left(\frac{S_i + S_{m_\infty}}{V_i + V_{m_\infty}}\right) - \ln\left[\left(\frac{S_i + S_{m_\infty}}{V_i + V_{m_\infty}}\right) - \left(\frac{S_i + S_{m_t}}{V_i + V_{m_t}}\right)\right] = Kt$$

in which S refers to amount of solute and V refers to volume. The subscripts

i and m refer to the interstitial space and mitochondrial space respectively in the whole pellet and ∞ and t refer to the appropriate values at equilibrium and time, t, respectively. The equation may be simplified to:

$$\ln\left[\left(\frac{S_i + S_{m_\infty}}{V_i + V_{m_\infty}}\right) - \left(\frac{S_i + S_{m_t}}{V_i + V_{m_t}}\right)\right] = -Kt$$

because the first logarithmic term is a constant and may be dropped for the reason described previously. If one assumes that S_i,V_i, and $V_{m_t} = V_{m_\infty}$ are constants, *i.e.*, a constant interstitial space, the equation can be simplified to:

$$\frac{\ln\left(\frac{S_{m_\infty} - S_{m_{t_1}}}{S_{m_\infty} - S_{m_{t_2}}}\right)}{t_2 - t_1} = K.$$

Thus the rate constant, K, is independent of the size of a constant interstitial space.

From the above reasoning and the fact that the experimental determination of K based either on whole pellet concentration changes or on mitochondrial concentration changes alone, results in approximately the same value for K, it would appear that the magnitude of interstitial space changes is small so that they do not affect the calculation of K based on whole pellet measurements.

The magnitude of the deviation at a time near zero of the curve obtained by plotting $\ln(C\infty - C_t)$ against time indicates that some cause in addition to mitochondrial volume increase is responsible. It may be that more than one type of mitochondria is present. If part of the mitochondria were permeable to the solutes studied while a second type were less permeable, there would result more than one rate constant for total permeation. Such a possibility is not unlikely, as visual inspection of a centrifugally isolated mitochondrial pellet reveals layers of differently colored mitochondria. Harman and Feigleson (22) have observed three types of rabbit heart mitochondria and Schneider (2) has reported that the layers seen in sedimented mouse liver mitochondrial pellets possess different enzyme activities.

An explanation for the differences in maximum volume attained by mitochondria in various media was not found in these experiments. It may be seen from the data given in Table II that experiments performed in 0.88 M sucrose resulted in less swelling than when mitochondria were incubated in other media. This occurred even though the concentration of sucrose inside the mitochondria had reached an equilibrium with the suspending medium sucrose concentration, thus ruling out a difference in osmotic pressure between the inside and outside owing to differences in sucrose concentration. The fact that mitochondria swell to large volumes when isolated in distilled water (1) is probably due to osmotically active molecules within the mitochondria,

some of which may slowly diffuse outward. It may be that differences between the rate of entering solute and internal osmotically active solute diffusing out of the mitochondria control the final volume, as has been shown to occur with oleate-treated erythrocyte ghosts (23). This does not, however, offer any simple explanation for the difference in maximum volume attained in 0.25 and 0.88 M sucrose.

Experiments similar to those of MacFarlane and Spencer (15) did not show that mitochondria could concentrate sodium against an external concentration gradient as reported by these investigators. A possible explanation for the discrepancy depends on the bound sodium content of the mitochondria. MacFarlane and Spencer measured the total sodium content of the mitochondria. In the same experiments they reported that mitochondria contained approximately 0.3 mg. of sodium per ml. of water when isolated in 0.25 M sucrose. The value represents bound sodium and one can subtract it from the total sodium concentration reported by MacFarlane and Spencer after incubation. The difference may be considered as the concentration of free diffusible sodium which has entered the mitochondrial pellet. These investigators found a maximum sodium concentration in the pellet at 60 minutes which was 1.17 mg. per ml. of mitochondrial water. Subtraction of 0.3 mg. per ml. for bound sodium results in a value of 0.87 mg. per ml. which is close to the 0.81 mg. per ml. of the suspending medium. The small discrepancy may be due to the uncertainty in the determination of the bound mitochondrial sodium in their experiment because in two separate experiments they reported values of 0.26 and 0.34 mg. per ml. for bound sodium. These considerations raise doubt about the ability of mitochondria to concentrate sodium against an external gradient *in vitro*, especially in view of the fact that both with the medium of MacFarlane and Spencer and with simple sodium chloride solutions the same sodium concentrations were reached in the mitochondrial pellet.

The reasoning is substantiated by the experiments of Spector (18), who showed that a medium at 37°C. containing AMP, sodium glutamate, cytochrome *c*, and phosphate-HCl buffer prevented a loss of bound potassium. In addition, with the same medium, to which was added potassium glutamate in concentrations of 0.01 and 0.04 M, Spector found no significant increase in bound mitochondrial potassium. In contrast to this, when mitochondria were incubated in isotonic sucrose at 37°C., the bound potassium was reduced to a value too small to measure, and sodium was reduced to 34 per cent of the value found in 0.25 M sucrose after 6 hours at 2°C. The reductions in sodium and potassium content occurred within 15 minutes when incubation was carried out at 37°C. Thus the fact that MacFarlane and Spencer found no increase in sodium concentration over that of the suspending medium when AMP was omitted may have been due to a loss of bound sodium.

It may be concluded from the experiments reported here that isolated

rat liver mitochondria show differences in permeability toward the various solutes studied. These experiments and the electron micrographs of Palade (3) and Dalton *et al.* (4) suggest that the mitochondria possess a membrane which regulates the rate at which various solutes can enter. Because the equilibrium concentrations reached in the mitochondrial pellets for various concentrations of sodium, potassium, and sucrose were observed to be close to that of the medium concentration, it would appear that most of the interior is available to these solutes.

Under the conditions of the present experiments it may also be concluded that movement into mitochondria of the solutes examined is controlled by a mitochondrial membrane exhibiting only passive permeability characteristics. It should be noted, however, that other investigators (15, 24) have postulated that under different conditions fresh mitochondria may be able to accumulate sodium and potassium against a concentration gradient in a process involving the expenditure of metabolic energy, and hence may be capable of the active transport of simple ions. On the other hand, it is becoming increasingly evident (16, 17, 24) that substantial amounts of sodium and potassium may be bound by mitochondrial constituents, and additional experimental data are needed to elucidate more fully the possible role of these ion-binding constituents in producing the discrepancies that are sometimes observed between intramitochondrial and extramitochondrial concentrations of sodium and potassium.

SUMMARY

The rates of penetration of various solutes into isolated rat liver mitochondria have been studied.

Sodium, potassium, and sucrose were observed to enter the mitochondria until an equilibrium concentration was reached. The diffusion of these solutes, after the first few minutes, followed the predicted diffusion curve for solutes entering a particle with a rate-limiting membrane and instantaneous mixing in the interior. Reasons for deviations from the predicted equation during the first few minutes of diffusion are suggested.

The data show that at pH 7.4 sodium and potassium enter more rapidly than sucrose. I^{131}-labelled albumin was found to enter very slowly, if at all. Increasing the pH from 7.4 reduced the rate at which sodium ion penetrated the mitochondria. The rate of diffusion of sucrose into mitochondria was considerably slower than diffusion of sucrose into a sphere of water of the same size.

Sodium ion was not found to be concentrated *in vitro* against an external concentration gradient as has been reported by other investigators.

It is concluded that the rate of diffusion of solutes between the external medium and the interior of mitochondria is probably restricted and controlled

by a mitochondrial membrane exhibiting passive permeability characteristics.

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INACTIVATION OF BACTERIA BY DECAY OF INCORPORATED RADIOACTIVE PHOSPHORUS*

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(Received for publication, February 10, 1956)

INTRODUCTION

Bacteriophages lose their infectivity upon decay of radiophosphorus P^{32} incorporated into their deoxyribonucleic acid (DNA). Each inactivation appears to be the consequence of the decay of a single atom of P^{32}, although only a fraction of such radioactive disintegrations is actually lethal. By means of reconstruction experiments it is possible to show that the ionizations produced by the hard P^{32} β-electrons on their way out of the virus particles are not the principal cause of this death, but that a "short range" consequence of the radioactive disintegration, like the transmutation $P^{32} \rightarrow S^{32}$ or the recoil energy sustained by the decaying phosphorus nucleus, must be responsible for the loss of infectivity (1). In the experiments reported here, similar lethal effects on the viability of bacterial cells containing P^{32} incorporated into their DNA have been observed. These findings reinforce the conclusions previously reached concerning the mechanism by which DNA macromolecules harboring decaying P^{32} atoms are destroyed (2). The localization of these lethal effects within the DNA of the bacterial cell, furthermore, makes possible certain experiments relevant to the function of the bacterial nucleus.

Materials and Methods

Bacterial Strains.—*Escherichia coli*, strain B/r, a radiation-resistant mutant (3), and *E. coli*, strain 15_{T^-}, a thymineless mutant (4), were employed in this study.

Media—H is a glycerol-lactate medium (2) and GCA a glycerol-casamino acid medium (5).

Colony Counts.—Aliquots of the bacterial cultures were spread on the surface of nutrient agar plates (6) and then incubated at 37°C. for 24 hours.

Radioactive Techniques.—The methods of preparation of radioactive growth media, measurement of radioactivity, and phosphorus determination were those previously described (2).

* Supported by grants from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, and The Rockefeller Foundation.

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Experimental

Inhibition of Bacterial Growth at High Specific Activities of P^{32}.—

Cultures of *Escherichia coli* will not grow when inoculated into media containing radiophosphorus P^{32} at specific activities superior to 500 mc./mg. That this inhibition of bacterial multiplication is produced principally by the decay of P^{32} atoms which have been assimilated into the cells and not by the radiation emitted by extracellular P^{32} atoms present in the growth medium has been demonstrated in the following way. A culture of *E. coli* was inoculated into 2 volumes of H medium, each containing 1.5 mc. of P^{32} per ml. but differing in total phosphorus content so that their specific radioactivities were 150 and 750 mc./mg. respectively. Periodic microscopic observation of the two cultures during incubation at 37°C. showed that bacterial division continued in the low specific activity medium, although some filaments were eventually produced. In the high specific activity medium, on the other hand, there was essentially no increase in the number of cells; many of the bacteria became filamentous and underwent lysis within 3 hours. The rate of P^{32} assimilation is proportional to the specific activity of the growth medium and hence the bacteria in the high specific activity medium incorporated initially many more atoms of P^{32} into their phosphorylated constituents per unit time than those in the low specific activity medium. Inasmuch as the growth media of both cultures contained exactly the same *total* radioactivity, it would follow that the selective inhibition of only the high specific activity culture was due to its higher content of assimilated P^{32} and concomitant greater frequency of intracellular disintegrations. A calculation shows that in each *E. coli* cell whose phosphorylated constituents contain P^{32} at the level of 500 mc. /mg. there will occur approximately ten radioactive disintegrations every minute. Hence it seems reasonable to suppose that the failure of cultures to multiply in the presence of very high specific activities of P^{32} is due to the fact that under these conditions many cells sustain a "lethal" disintegration before they have had time to go through one division cycle. The following experiments, in which the lethal effects of the decay of incorporated P^{32} on *E. coli* bacteria have been examined in more detail, substantiate this supposition.

Inactivation of Bacteria by Decay of Incorporated P^{32}.—

The bactericidal effect of the decay of incorporated P^{32} has been studied by storing radioactive bacteria at −196°C. in a frozen, *i.e.* metabolically inert state. The ability of individual cells to give rise to a colony after various amounts of radioactive decay had occurred was then tested by thawing and plating aliquots of the frozen culture from time to time. All those cells were considered to have been "inactivated" which failed to give rise to a visible colony upon being plated.

H medium, containing P^{32} of specific activity 16 mc./mg., was inoculated with a culture of strain B/r already in its exponential phase of growth in non-radioactive H medium and incubated at 37°C. until the bacterial concentration had increased from 2×10^6 to 10^8 cells/ml. The radioactive culture was then diluted into cold GCA medium to reduce the concentration of radioactivity to 0.01 mc./ml.(storage

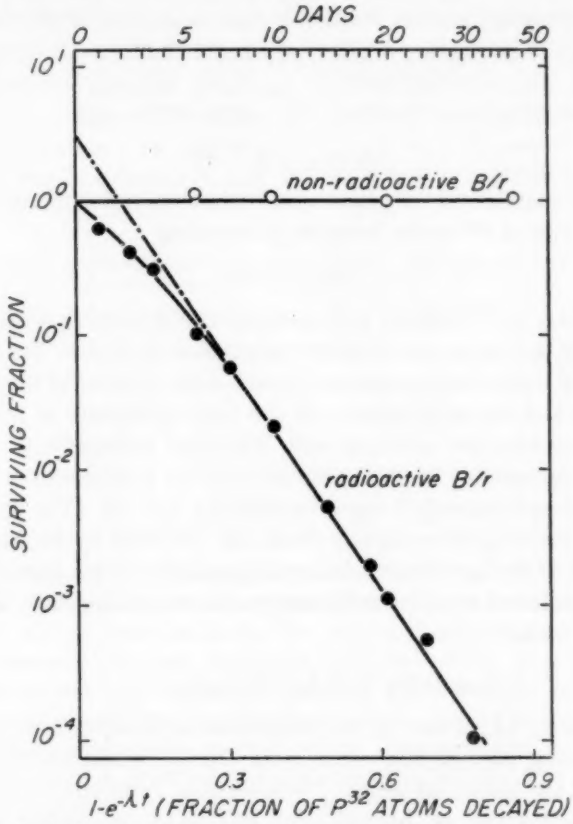

FIG. 1. Survival of radioactive (A_0 = 16 mc./mg.) and non-radioactive cultures of *E. coli* B/r at −196°C.

tube). 0.1 ml. aliquots of the storage tube were then frozen and stored in liquid nitrogen (−196°C.). From day to day, one of the frozen aliquots was thawed and plated for colony counts. A non-radioactive control culture of B/r was diluted into GCA medium (to which 0.01 mc./ml. P^{32} had been added) and similarly frozen, stored, and assayed. At least 90 per cent of the bacterial cells were recovered as colony formers when a culture was thawed and plated immediately after being frozen.

The results of this experiment are presented in Fig. 1, on which the logarithm of the fraction of bacteria surviving as colony formers has been plotted against

the fraction of P^{32} atoms decayed at the time of assay. It is apparent that the number of non-radioactive cells capable of forming colonies remained constant, *i.e.* that bacterial survival appeared to be affected neither by prolonged storage at −196°C. nor by any irradiation produced by the low level of unincorporated P^{32} present in the storage tube. Among the radioactive bacteria, on the other hand, a progressively smaller fraction is seen to survive, so that less than 1 in 10^4 is capable of generating a colony when 0.8 of the incorporated P^{32} atoms has decayed. The surviving fraction, s_n, of the radioactive bacteria seems to disappear according to a "multiple hit" curve of the type

$$s_n = 1 - (1 - s)^n, \tag{1}$$

as if each cell contained n "sensitive units" whose surviving fraction s decreased with the fraction of P^{32} atoms decayed, f, according

$$\log s = -Kf \tag{2}$$

in which K is a rate constant, and as if the colony-forming ability of the cell was preserved as long as one of these units remained intact. The final slope K of the survival curve then represents the rate of inactivation of the last sensitive unit per cell and the extrapolation of the final asymptote to zero decay the number, n, of sensitive units per cell. The final asymptote of the survival curve of the radioactive bacteria presented in Fig. 1 extrapolates to the value 3, indicating approximately 3 sensitive units per B/r cell. (The actual shape of the survival curve deviates slightly from that described by Equation 1; this is probably due to the fact that the bacterial population is not homogeneous with respect to number of sensitive units, some cells containing more and some less than 3 units each.)

Inactivation of Differentially Labeled Bacteria.—

Approximately 12 per cent of the phosphorus of *E. coli* cells resides in DNA, the rest forming part of ribonucleic acid (RNA), phospholipides, phosphoproteins, and low molecular weight ("acid-soluble") compounds. If the mechanism of inactivation of bacteria by P^{32} decay is similar to that by which radioactive bacteriophages lose their infectivity, it is possible that only the decay of radioactive phosphorus atoms incorporated into the bacterial DNA is responsible for loss of colony-forming ability and not the decay of the majority of the intracellular P^{32} atoms residing in the other phosphorylated cell constituents. The following experiments with *E. coli*, strain 15_{T^-}, a thymine-requiring mutant, show this to be the case. If thymine is removed from the growth medium of 15_{T^-}, the number of viable bacteria in the culture remains constant for a period roughly equivalent to one-half the normal generation time and then decreases rapidly ("thymineless death"). During such thymine starvation, the bacteria increase in size, double their content of RNA but in-

crease in their DNA by no more than 5 to 20 per cent (4, 7). The preferential reduction of DNA synthesis in the absence of exogenous thymine thus provides a means by which relatively more P^{32} can be introduced into other cellular constituents than into DNA. The rate of inactivation of cultures which contain similar *total* amounts of P^{32} per cell but different specific activities in their DNA can then be compared.

A culture of 15_{T^-} growing in H medium supplemented with thymine was washed free of thymine and incubated for 10 minutes at 37° in thymine-free H medium. Two aliquots of radioactive H medium of specific P^{32} activity 280 mc./mg., one containing no thymine and one containing 5 mg./ml. thymine, were then inoculated with these bacteria to a density of 5×10^7 cells/ml. After incubation at 37° for 2

TABLE I

Assimilation of P^{32} by E. coli 15_{T^-} in the Presence and Absence of Thymine

Growth medium (A_0 = 280 mc./mg.)	Time of incubation	Relative titer of viable bacteria	TCA-insoluble P^{32}
	min.		*cts./min./ml. of culture*
With thymine	2	1.0	6.6×10^6
	30	1.2	2.2×10^6
Without thymine	2	1.0	6.0×10^6
	30	0.6	1.8×10^6

and 30 minutes, samples of both cultures were diluted into cold GCA medium (storage tubes). The storage tubes were then frozen, stored, and thawed and plated from day to day for colony formers, as above. The amount of radiophosphorus assimilated by the two cultures during their incubation in the radioactive H media was measured as trichloroacetic acid (TCA)–insoluble P^{32}. For this purpose, an aliquot of each storage tube was added to an equal volume of cold 0.6 M TCA, containing 2×10^8 non-radioactive carrier bacteria per ml. Unincorporated and TCA-soluble P^{32} were then removed by centrifugation and washing in the cold. The TCA-insoluble material was dissolved in 1 N NaOH and its content of P^{32} counted.

The results of this experiment are presented in Table I where it is seen that the titer of viable bacteria in the radioactive culture containing thymine increased by 20 per cent between the 2nd and 30th minute of incubation whereas the titer of viable bacteria in the thymine-free culture decreased by 40 per cent during the same time. This difference in final titer reflects, on the one hand, the growth of the culture in the presence of thymine and, on the other hand, the onset of "thymineless death" in the absence of thymine. The amount of TCA-insoluble P^{32} which the one culture had assimilated in the absence of thymine was only 20 per cent less than that which the other had assimilated in the presence of thymine. The survival after P^{32} decay of the two cultures incubated

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for 30 minutes is presented in Fig. 2 *a*, on which the logarithm of the fraction of individuals capable of generating a colony after thawing is plotted against the fraction of P^{32} atoms decayed. It is seen, first of all, that although the time

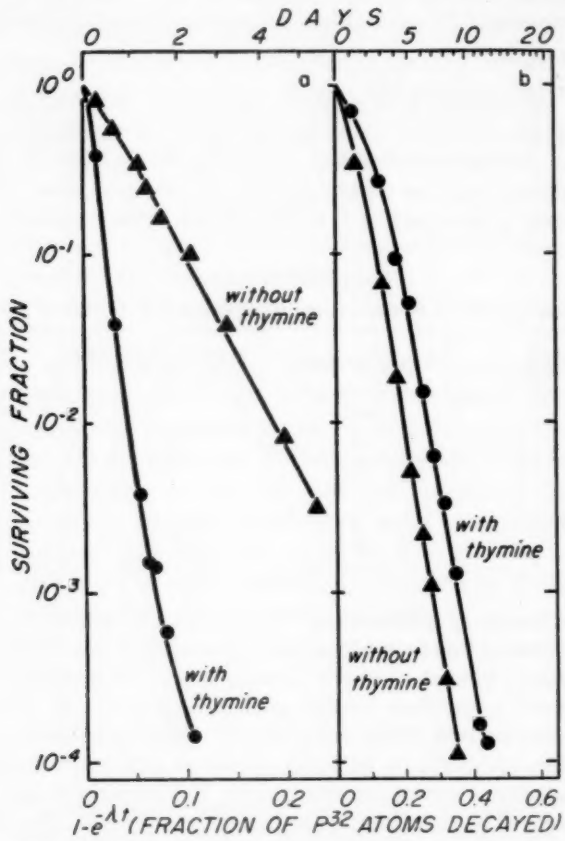

FIG. 2 *a*. P^{32} inactivation of *E. coli* 15_T- at −196°C. after incubation for 30 minutes in radioactive media (A_0 = 280 mc./mg.) with and without thymine. *b*. Effect of thymine starvation on P^{32} inactivation of 15_T- (A_0 = 16 mc./mg.) at −196°C. Triangles, radioactive bacteria subjected to 40 minutes' thymine starvation in radioactive medium (A_0 = 16 mc./mg.) prior to storage. Circles, radioactive bacteria incubated for 40 minutes in radioactive medium (A_0 = 16 mc./mg.) with thymine before storage.

of incubation of both cultures was only 30 minutes, more than 99 per cent of the cells of both cultures are subject to inactivation by P^{32} decay. The culture which had assimilated its P^{32} in the presence of thymine, however, is inactivated much more rapidly than that which had assimilated its P^{32} in the absence of exogenous thymine. In fact, the maximum slope of the inactivation curve of

the former is approximately 4 times greater than that of the latter, although, as is evident in Table I, the cells of both cultures contain practically equal *total* amounts of P^{32}.

It is conceivable that the great difference in rate of inactivation of the two cultures presented in Fig. 2 *a* could be due to some physiological effect produced by the 30 minute thymine starvation to which one of the cultures was subjected, an effect which might be thought to have ***reduced*** the ***inherent sensitivity*** of the 15_T-bacteria to inactivation by decay of assimilated P^{32}. To test for this possibility, a culture of 15_T- was grown in thymine-supplemented, radioactive H medium containing P^{32} at 16 mc./mg. After a 50-fold multiplication over the inoculum had occurred, the bacteria were washed free of thymine and 2 aliquots incubated for 40 minutes at 37° in H medium of the original specific activity, one in the presence and the other in the absence of thymine. Both aliquots were then diluted in cold GCA medium (storage tube). The storage tubes were then frozen, stored, and thawed and plated from day to day for colony formers as above.

The results of this experiment are presented in Fig. 2 *b*, in which it may be seen that the initial rate of inactivation of the thymine-starved culture, was, in fact, greater, not less, than that of the culture which had never been deprived of thymine. The final rates of inactivation of both cultures however, are seen to be identical. It would appear, therefore, that the fourfold difference in the rate of inactivation of the two cultures presented in Fig. 2 *a* is not due to a reduction of the P^{32}-sensitivity produced by thymine starvation. The reduced rate of inactivation of the cells which had assimilated their P^{32} in the *absence* of thymine must, rather, be due to the fact that very little of this P^{32} was introduced into the bacterial DNA. Consequently, it may be inferred that it is the decay of the DNA–radiophosphorus atoms which is mainly responsible for the loss of the colony-forming ability of the bacterial cells. It may be concluded, furthermore, that, as in the case of the P^{32} inactivation of bacterial viruses, a "short range" effect of the radioactive disintegration is responsible for death and not the β-radiation concomitantly emitted. For the range of the β-electrons emitted by P^{32} is so much longer than the dimensions of the *E. coli* cell, that were β-ionizations mainly responsible for bacterial death, no difference in lethal efficiency could exist between β-electrons originating in the DNA and those originating in other cellular constituents. The fact that the thymineless culture of Fig. 2 *a* is inactivated at all is compatible with the idea that this inactivation is due to those P^{32} atoms which found incorporation into DNA in the course of the small ***residual*** DNA synthesis which may take place even in the absence of thymine (4).

Efficiency of Killing by P^{32}.—

The lethal P^{32} disintegrations are thus seen to be mainly those which take place in the DNA, *i.e.* in the bacterial nucleus, the seat of the bacterial DNA

(8, 9). It has been observed in cytological studies that, depending on the conditions of growth, the "most frequent" number of nuclei possessed by cells of strain B/r is between 2 and 4 (3). It would seem, therefore, that the 3 sensitive units per cell inactivated separately by P^{32} decay in the radioactive B/r culture of Fig. 1 are the bacterial *nuclei*. Each cell appears to be capable of giving rise to a colony as long as P^{32} decay has left at least one of its nuclei intact. The final slope K of the inactivation curve of Fig. 1 then represents the rate of inactivation of individual nuclei by decay of P^{32} atoms they contain. It can be shown (1) that the rate constant K has the value

$$K = 1.48 \times 10^{-4} \alpha A_0 N \tag{3}$$

in which A_0 is the initial specific activity of the DNA–P^{32}, N the number of DNA–phosphorus atoms per nucleus, and α the "efficiency of killing," *i.e.*,

TABLE II

Estimation of α for E. coli B/r at −196°C.

Material	A_0	Final slope of death curve, K	DNA-P per cell	Nuclei per cell	DNA-P per nucleus	N atoms of DNA-P per nucleus or phage	α
	mc./mg.		*mg.*		*mg.*		
B/r	16	−8.3	180×10^{-14}	3	60×10^{-14}	120×10^3	0.02
	45	−17					
	92	−33					
T3	160	−1.6				2×10^3	0.04

the fraction of the DNA–P^{32} disintegrations which are actually lethal to the nuclei in which they take place. In order to calculate the magnitude of α for P^{32} disintegrations within B/r, N, the number of phosphorus atoms per nucleus of the B/r cultures employed in these experiments has been estimated by assaying the DNA–phosphorus content per cell by means of the Schmidt-Thannhauser method (10) and dividing this value by 3, the number of sensitive units per cell.

The results of these determinations and calculations are presented in Table II, in which not only the final slope K of the inactivation curve of the radioactive B/r of Fig. 1 but also the final slopes of two other inactivation experiments with more highly radioactive B/r cultures are listed. It is seen that, as demanded by equation (3), the rate of inactivation of individual nuclei K in the three experiments is roughly proportional to the specific activity A_0. The final column lists the efficiency of killing α, estimated on the basis of the three inactivation experiments, which is seen to be 0.02 "hits" per P^{32} disintegration per nucleus. In other words, *one out of every fifty P^{32} disintegrations taking place at −196° appears to inactivate the B/r nucleus in which it has occurred.* For

comparison, the data for an efficiency of killing calculation for the coliphage strain T3 are also presented in Table II, for which α is seen to be 0.04 "hits" per P^{32} disintegration per phage. The efficiencies of killing in these two rather different biological objects are, therefore, seen to be of the same order of magnitude.

On the basis of equation (3) and the values of the rate constants K given in Table II, one may now estimate that in B/r cells containing more than 500 mc./mg. P^{32} there should be on the average more than one lethal disintegration per nucleus every hour, the division time of strain B/r in radioactive H medium at 37°. Since the efficiency of killing could be expected to be even greater at 37° than at −196° (2), it is apparent that *E. coli* cultures are indeed no longer able to multiply at high specific activities because most of the cells suffer inactivation before having had time enough to reproduce themselves.

Multiplication and Thymineless Death of Bacteria after P^{32} Decay.—

It has been seen that a number of nuclei must apparently be inactivated by P^{32} decay in each bacterium before the ability of the cell to give rise to a clone of descendants, *i.e.* to a colony, is finally destroyed. Two further properties of 15_T- populations have now been examined at various stages of P^{32} decay: (*a*) the multiplication of the survivors in a medium which contains thymine and (*b*) the inactivation of the survivors by thymine starvation in a thymine-free medium (thymineless death).

Two cultures of 15_T- were grown in thymine-supplemented H medium, one in the presence of P^{32} at 16 mc./mg. and the other in the absence of any P^{32}. After growth, the cultures were diluted into cold GCA medium and the storage tubes frozen, stored, and thawed and plated from day to day for colony formers, as above. At various stages of P^{32} decay, furthermore, thawed aliquots of both radioactive and non-radioactive cultures were diluted either into H medium containing 2 γ/ml. thymine or into thymine-free H medium, then incubated at 37° and plated for colony formers after various times of incubation.

The results of this experiment are presented in Fig. 3 *a* and 3 *b*, on which the fraction of the original number of cells in each culture capable of giving rise to a colony is plotted against the time of incubation at 37°. The intercept of each curve with the ordinate of zero incubation time in the two figures represents the fraction of the original population "surviving" the amount of P^{32} decay which had taken place by the day on which the sample was thawed. It is seen in Fig. 3 *a* that the non-radioactive culture when thawed either soon after being frozen or after being stored for 3 or 6 days resumed its multiplication without any appreciable delay when transferred to 37° in the presence of thymine. In other words, immediately after thawing, the bacteria appear to resume their activities where they had left them off at the moment of freezing. The

radioactive culture, on the other hand, already exhibited a 40 minute lag before multiplication resumed when it was thawed after only a few hours' storage at −196°, at which time there had been little or no inactivation of its colony-forming ability. When the radioactive culture was thawed after 3 days' storage,

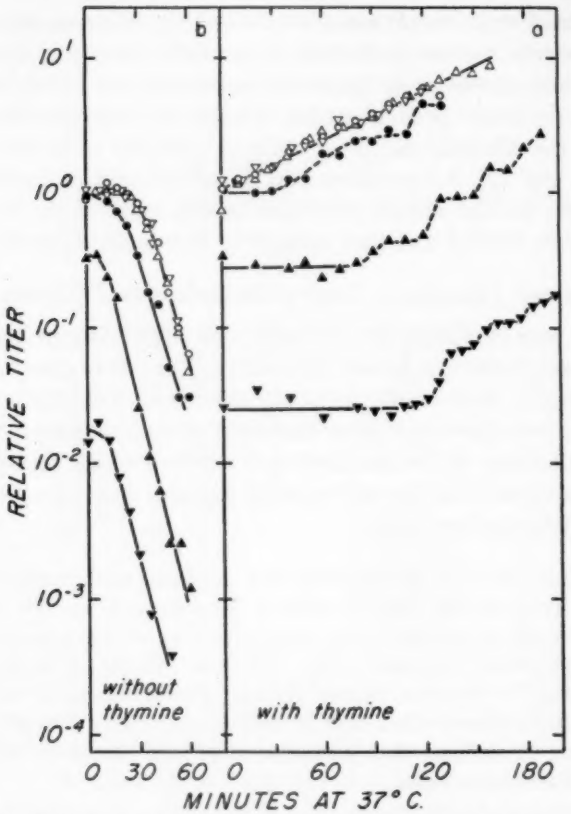

FIG. 3 *a*. Multiplication in thymine-supplemented medium and *b* thymineless death in thymine-free medium of radioactive and non-radioactive cultures of *E. coli* 15_T- after storage for various times at −196°C. Time of storage: circles < 0.5 day; upward triangles, 3 days; downward triangles, 6 days. Filled symbols, radioactive culture (A_0 = 16 mc./mg.); open symbols, non-radioactive culture. Ordinate, colony counts relative to original bacterial population. Abscissa, time of incubation at 37°C. after thawing.

when 30 per cent of the initial population was still capable of colony formation, the division lag of the survivors increased further to 80 minutes. Finally, after 6 days' storage, when only 2 per cent of the radioactive 15_T- cells were still capable of giving rise to a colony, 110 minutes at 37° had to elapse before the thawed culture manifested any signs of multiplication.

The multiplication lag of the bacteria surviving P^{32} decay reflects some "non-lethal" damage wrought by the intracellular radioactive disintegrations. Since in the course of P^{32} decay the number of intact nuclei per surviving cell is thought to be reduced until there remains only one, it would appear reasonable to attribute some of the observed multiplication lag to the time required for the cell to regenerate a sufficient number of functional nuclei with which viable daughter cells can be endowed. It is difficult, however, to explain entirely on this basis the further increase in lag from 80 to 110 minutes while the fraction of P^{32} survivors decreases from 0.3 to 0.02, since this part of the inactivation is already proceeding according to the "exponential" part of the multiple hit survival curve, when the surviving bacterial population should finally be "mononucleate." Possibly the necessity for repair of some additional non-lethal, cytoplasmic damage is responsible for this further increase in lag. It should be noted that the multiplication of the survivors of P^{32} decay appears to take place in a stepwise manner, not unlike the "synchronous" growth of 15_T- observed among the survivors of short periods of thymine starvation (11). The possibility of achieving synchronous growth by P^{32} decay has, however, not been examined with sufficiently great precision to make this observation statistically significant.

The effect of thymine starvation on radioactive and non-radioactive cultures after various lengths of storage at $-196°$ is presented in Fig. 3 *b*. It is seen that the three aliquots of non-radioactive 15_T- cells stored in the frozen state for various times all exhibit the typical "thymineless death" upon growth in thymine-free medium, *i.e.* constant colony count for the first 25 minutes followed by rapid loss of colony formers, leaving only 10 per cent of the initial population viable at the 55th minute. The survivors of the radioactive 15_T- cultures are likewise seen to be subject to thymineless death, but it is to be noted that the period of constant cell count appears to be *reduced* and the onset of loss of colony formers hastened by prior P^{32} decay. In the culture subjected to only a few hours of P^{32} decay, thymine starvation for 45 minutes left 10 per cent of the initial colony formers surviving. In the cultures stored for 3 and 6 days, in which P^{32} decay had left respectively 30 and 2 per cent survivors, thymine starvation for only 30 minutes following thawing sufficed to reduce the count of viable cells by another factor of ten. The *rate*, however, at which thymineless death proceeds, once under way, appeared to be unaffected by any previous P^{32} decay. If the result presented in Fig. 2 *b* is recalled, in which it was observed that *prior thymine starvation* hastened the initial rate at which 15_T- cells are then inactivated by P^{32} decay, though leaving the ultimate or exponential part of the P^{32} survival curve unaffected, it becomes evident that P^{32} decay and thymine starvation *cooperate* in bringing about the death of bacterial cells as colony formers. A preliminary treatment by one reduces the amount of subsequent treatment required to produce the maximum inactivation rate by the other. Since the lethal effect of P^{32} decay has been localized

within the DNA, it seems plausible to infer from these observations that the lethal effect of thymine starvation is likewise an inactivation of bacterial nuclei. When, therefore, either P^{32} decay or thymine starvation has already inactivated some of the nuclei of a surviving 15_T- cell, subsequent starvation of or decay in such a population will eliminate the remaining intact nuclei, and hence the ability of the cell to give rise to a colony, after a shorter induction period. It should be emphasized here that the actual mechanism by which the bacterial nucleus is affected and its physiological and biochemical potentialities after inactivation could be entirely different following the two types of treatment. Required under this view is only that a nucleus "inactivated" in either manner is no longer capable of regenerating an indefinite succession of daughter nuclei for a clone of viable descendants.

Distribution of the DNA–Phosphorus Atoms of Bacteria among Daughter Cells.—

It has been shown that the DNA of *E. coli* cells retains its phosphorus atoms throughout subsequent bacterial growth and multiplication (12, 13). How are these phosphorus atoms distributed over the nuclei of daughter cells? Do some descendant nuclei contain only atoms assimilated *de novo* and are others endowed exclusively with phosphorus atoms of parental origin, or are the atoms of the parental nucleus dispersed among all the nuclei in its line of descendance? The fact that it is the decay of DNA–P^{32} atoms which is mainly responsible for the death of the bacterial cells offers a method of resolving this question. For if in the course of their reproduction the parental nuclei preserve their atomic integrity, *i.e.* if newly assimilated phosphorus atoms are introduced only into the progeny structures, then the stable nuclei of a non-radioactive culture of cells inoculated into a medium containing P^{32} would never acquire any radioactivity and hence remain stable. After growth in the radioactive medium, the culture would still possess its initial number of non-radioactive nuclei and the colony-forming ability of cells harboring these nuclei could not be eliminated by P^{32} decay. Conversely, when a radioactive culture, homogeneously subject to loss of colony-forming ability by radioactive decay, is inoculated into a medium containing no P^{32} and if the nuclei subsequently synthesized contain only *de novo* material, then very quickly a class of cells will arise which contain entirely non-radioactive, *i.e.* stable nuclei and which should be refractory to inactivation by decay. If, on the other hand, the atomic identity of the nucleus is destroyed in the course of bacterial proliferation and the parental atoms dispersed among the daughter structures, then in the two experiments just considered stable nuclei of non-radioactive cells will quickly become radioactive upon growth in the presence of P^{32} and radioactive nuclei will still give rise to radioactive, albeit *less* radioactive, nuclei upon growth in the absence of P^{32}.

H medium containing P^{32} at 200 mc./ml. was inoculated with growing, non-radioactive B/r bacteria to a final density of 10^8 cells/ml. This radioactive culture was

incubated at 37° and from time to time aliquots diluted into cold GCA medium (storage tubes). The cell concentration in the radioactive culture was maintained near 10^8 per ml. by intermittent dilution with appropriate volumes of fresh H medium containing P^{32} at the original specific activity. When a 20-fold multiplication of the inoculum had taken place, an aliquot of the radioactive culture was diluted into an equal volume of non-radioactive H medium containing enough inorganic phosphate to reduce the specific activity of the P^{32} introduced by radioactive culture

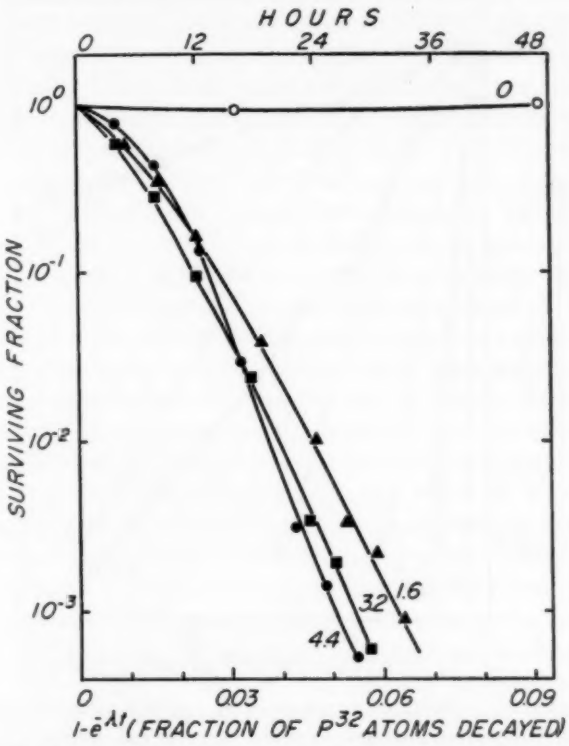

FIG. 4. Survival at −196°C. of a culture of *E. coli* B/r after various numbers of divisions in radioactive medium (A_0 = 200 mc./mg.). Parental bacteria non-radioactive.

to 4 mc./mg. After this reduction of specific P^{32} activity, the culture was further incubated at 37° and aliquots diluted into cold GCA medium from time to time, while the cell concentration was maintained near 10^8 per ml. by intermittent dilutions into non-radioactive H medium. The storage tubes were frozen, stored, and thawed and assayed for colony formers as above.

The results of the first part of this experiment are presented in Fig. 4, in which the survival of the non-radioactive B/r culture, which has undergone various numbers of divisions in radioactive growth medium, is shown as a

function of the fraction of P^{32} atoms decayed. The parental culture, having not yet made any divisions, is seen to be stable. After 1.6 divisions in the presence of P^{32}, however, at least 99.9 per cent of the bacterial cells had become sensitive to inactivation by P^{32} decay. After 3.6 and 4.4 divisions, the populations are

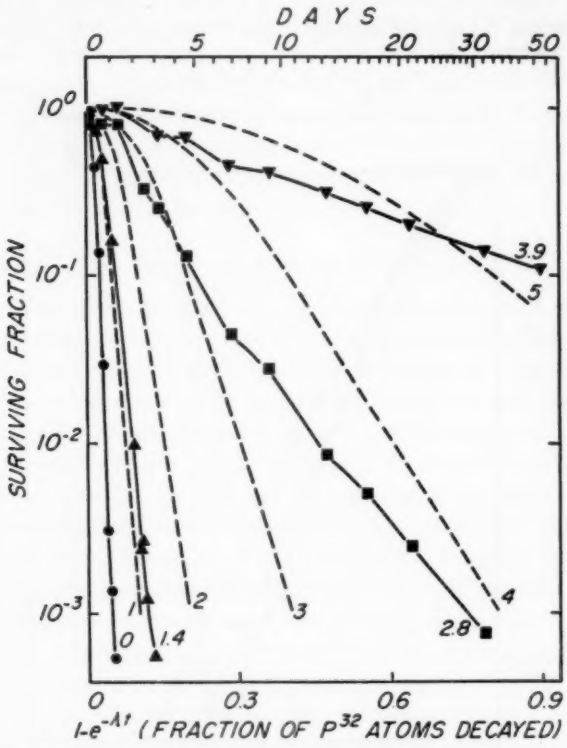

FIG. 5. Survival at −196°C. of a culture of *E. coli* B/r after various numbers of divisions in non-radioactive medium. Parental bacteria radioactive (A_0 = 200 mc./mg.). The dashed curves indicate the expected survival of the descendants after D divisions if at each division the parental DNA is equipartitioned among the two daughter cells, calculated according to

$$s_n(D) = 1 - [1 - \exp(-Kf/2^D)]^n.$$

seen to have experienced a further increase in sensitivity to inactivation. The slope of the exponential part of the inactivation curve after 1.6 divisions is 70 per cent of the final slope after 4.4 divisions, in harmony with the estimate that after 1.6 divisions approximately 66 per cent of the bacterial DNA has been synthesized *de novo*. It, therefore, appears that *the atomic identity of the bacterial nucleus is not preserved in the course of its reduplication* but that *parental and newly assimilated DNA–phosphorus atoms become intermingled within*

daughter nuclei. The results of the second part of this experiment are presented in Fig. 5, on which the survival of the radioactive B/r culture, which have undergone various numbers of divisions in non-radioactive medium, is plotted against the fraction of P^{32} atoms decayed. The parental culture, having not yet undergone any divisions in the absence of P^{32} and being identical to the 4.4 divisions culture of Fig. 4, is seen to be subject to rapid death. The cultures having undergone 1.4, 2.8, and 3.9 divisions respectively are seen to have become progressively less sensitive to inactivation, but in none of these populations had there appeared a detectable fraction of completely stable cells. The survival of the culture after various numbers of divisions, expected on the basis of an equipartition of the parental atoms at each nuclear duplication has also been indicated in Fig. 5. These theoretical survival curves were calculated by a "dose-reduction" principle, *i.e.*, by multiplying the abscissae of the survival curve of the original radioactive parental population by the factor by which the number of cells in the culture had increased over the inoculum. A comparison of the observed with the theoretical survival curves shows that after 1.4 divisions the population is inactivated as though the P^{32} of the parental DNA is equidistributed among daughter nuclei. After 2.8 and 3.9 divisions, however, the survival curves depart more and more from those predicted by equidistribution. It is possible that this departure is the reflection of the appearance of an asymmetry in the partition of the DNA phosphorus atoms during the second but not during the first nuclear reduplication following their assimilation. An equally likely explanation would be, however, that the slowly dying tail of the later survival curves represents the descendants of a more rapidly growing minority of the bacterial population, which had undergone more divisions than the average and had, therefore, reduced its specific radioactivity content more than estimated by the dose-reduction calculation. In any case, the second part of this experiment confirms the conclusion already reached from the results of the first, *i.e.*, that the phosphorus atoms of the parental nucleus become dispersed among its descendants. This conclusion is in harmony with the observation that the phosphorus atoms of the DNA of T2 and T4 bacteriophage particles are likewise dispersed, though not equidistributed, over their progeny (14, 15). It is, unfortunately, not possible to infer from the present experiments the mechanism by which this dispersion occurs, *i.e.* whether it is due to a partition of the parental atoms in the course of the elementary replication act of the DNA itself, such as demanded by some proposals concerning this process (16, 17) or whether it is due to the randomizing effect of some postreplication event, such as "crossing-over" or assortment of "chromosomes."

DISCUSSION

It has been observed that the efficiency of killing α with which decay of incorporated P^{32} atoms destroys the infectivity of bacteriophage particles is

very nearly the same for a number of bacteriophage strains which differ greatly in their size, chemical constitution, morphology, genetic structure, and manner of interaction with bacterial host cells. This efficiency on the other hand varies with the temperature at which decay proceeds, rising from 0.04 at $-196°$ to 0.3 at $+65°$ (2, 18, 19). On the basis of these findings it was concluded that the efficiency α must reflect some structural aspect of the DNA macromolecule rather than of the virus particles. A mechanism was suggested in terms of the two-stranded helical Watson-Crick structure of DNA (20) to account for the manner in which decay of incorporated P^{32} affects the integrity of the molecule harboring the disintegrating atoms. It was proposed that the high proportion of non-lethal decays reflected the possibility that the physiological function of the DNA molecule could be preserved even after radioactive decay had interrupted one of the single polynucleotide strands. The lethal decays were thought to be only those which had resulted in a complete cut of a DNA double helix (2). The discovery that one "lethal" P^{32} disintegration suppresses the survival of only a part of the genome of an inactivated bacteriophage particle has lent further support to the notion that the lethal effect is localized within a single macromolecule (21, 22).

The finding reported here that bacterial nuclei appear to be inactivated by decay of incorporated P^{32} with an efficiency of killing of the same order of magnitude as bacteriophages strengthens these views concerning the mechanism of damage. For the great difference in size, structure, and function which must exist between *E. coli* nuclei and bacteriophages makes it unlikely that any factor other than the similarity of the basic structure of the DNA molecules of both could be responsible for so comparable a fraction of the P^{32} disintegrations being lethal. (The difference between the efficiency α actually found in B/r (0.02) and that observed for T3 (0.04) at the same temperature, cannot be considered very significant, in view of the possible multiplicative errors inherent in the determinations of total amount of DNA phosphorus per cell, number of "sensitive units" per cell, and specific activity of growth media. This discrepancy might, nevertheless, be the reflection of some genuine structural difference.) The present observations, finally, confirm the earlier conclusions that β-electron ionizations are not mainly responsible for the lethal effects of incorporated P^{32}.

The two applications of P^{32} decay in bacteria presented here, *i.e.* the mechanism of "thymineless death" and the partition of parental DNA phosphorus atoms among daughter cells, would appear to be only examples of a larger class of experiments susceptible of exploitation by this technique. Many other problems in which it is desired to investigate the role of the bacterial nucleus in some aspect of metabolism or physiology could possibly be resolved by examination of radioactive bacteria before and after P^{32} decay. Such experiments concerned with the capacity of bacteria to reproduce infecting bacteriophages

or to synthesize induced enzymes have already been carried out and will be reported elsewhere.

We wish to thank Dr. W. M. Stanley for his interest in this work and Dr. S. S. Cohen and Miss Hazel D. Barner for kindly supplying us with strain 15_{T^-}, offering valuable advice concerning its culture, and acquainting us with their unpublished observations.

SUMMARY

Cultures of *Escherichia coli* will not grow in media containing very high specific activities of radiophosphorus P^{32}, the inhibition of growth being due to the decay of assimilated P^{32} atoms. Experiments with a differentially labeled thymineless strain of *E. coli* show that the P^{32} disintegrations which occur in the bacterial deoxyribonucleic acid, *i.e.* in the nucleus, are mainly responsible for the inactivation of the cell. The kinetics with which radioactive bacterial populations are inactivated indicate that the function of several nuclei per bacterial cell must be eliminated by P^{32} decay before the ability to generate a colony is lost. The efficiency with which each P^{32} disintegration inactivates the nucleus in which it has occurred is calculated to be 0.02 (at $-196°$), *i.e.*, similar in magnitude to the killing efficiency of P^{32} decay in bacteriophages.

P^{32} decay and thymine starvation cooperate in bringing about the death of individuals of the thymineless strain, from which observation it is inferred that "thymineless death" is likewise a nuclear inactivation.

The descendants of a non-radioactive bacterial culture grown for several generations in the presence of P^{32} and the descendants of a radioactive culture grown in the absence of P^{32} are inactivated by P^{32} decay in a manner which indicates that the phosphorus atoms of bacterial nuclei are dispersed among the progeny nuclei in their line of descendance.

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PRESSURE-VOLUME RELATIONSHIP OF THE FUNDULUS EGG IN SEA WATER AND IN SUCROSE*

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(Received for publication, March 7, 1956)

INTRODUCTION

The fact that the eggs of some teleosts, *Fundulus* (4, 5), trout (1, 6, 13), and salmon (12) possess a plasma membrane which is impermeable to water remains an enigmatic problem. Because of the absence of D_2O exchange under conditions of osmotic filtration and diffusion, Ussing (15) suggested that the plasma membrane of the trout egg was composed of a continuous lipide phase containing no pores, and allowing no solution of water molecules. Experiments in which pressure and volume were measured simultaneously and continuously have shown that the impermeable quality of the plasma membrane of *Fundulus* egg occurs only in activated or fertilized eggs, and that it is acquired during the activation process (5). This has been confirmed for the salmon egg by studies of D_2O exchange (12). The problem of interest which prompted the experiments reported herein concerns the changes which occur during activation and which ultimately lead to the impermeable state of the plasma membrane.

Some features of activation in the *Fundulus* egg have been described previously (4), and except for minor details, appear to be the same for other teleost eggs. Briefly, the outer enclosing chorion of the *Fundulus* egg becomes inelastic shortly after contact with sea water, and thereafter maintains a constant volume. Upon successful insemination or activation, numerous small vesicles in the peripheral aspects of the egg proper rapidly disintegrate and appear to release some colloidal material between the chorion and the egg proper. Upon imbibition of water, the colloid swells and causes the formation of a perivitelline space which separates the chorion from the egg proper. Two

* The work was carried out at Woods Hole, in the summer of 1954, in a laboratory provided for Dr. H. Grundfest (Department of Neurology, Columbia University, College of Physicians and Surgeons, New York), by a grant from the Marine Biological Laboratory under its ONR contract (Nonr-09703). I am indebted to Dr. Grundfest for the facilities and to the Director and staff of Marine Biological Laboratory for their cooperative assistance.

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other distinct features accompany the gradual growth of the perivitelline space. Since the chorion is inelastic, the perivitelline space enlarges at the expense of the egg proper which decreases in volume and surface area. Also, a relatively high colloid osmotic pressure, 150 mm. Hg when stabilized, is formed in the perivitelline space, and is transmitted throughout the entire egg enclosed within the chorion.

It has been suggested (4, 5) that the shrinking of the egg proper is related to the increasing perivitelline pressure. The purpose of this paper is to describe some experiments by which the pressure-volume relationship can be evaluated. In order to strengthen the evidence obtained from studying the normal progression of pressure increase, the events were modified or reversed by immersing the eggs in a hypertonic sucrose solution. In sucrose, because of a slow rate of penetration through the chorion, the pressure and volume changes were sufficiently slow to allow both to be measured. In addition, the degree of the changes produced also served as a severe test of the pressure-volume relationship.

Material and Method

As in previous experiments, pressure and volume changes were followed simultaneously on the same unfertilized egg activated by puncturing with a micropipette. The details of the method and apparatus have been described elsewhere (4). In essence, intracellular pressure was measured with a mercury manometer connected to a micropipette which was inserted deep into the interior of an egg. Volume was determined by measuring the diameter of the spherical egg. In most experiments, internal pressure was allowed to develop to its full extent after activation in sea water. When pressure was stabilized, the external sea water was removed, and a known volume of a test solution of sucrose was added. The volume of the test solution was large enough compared to the amount of sea water adhering to the chorion and its jelly strands, so that no significant dilution occurred after mixing. The concentrations of sucrose ranged from 0.01 M to 1.9 M, and were usually made up in sea water so as to avoid the complications of salt dilution. Occasionally, test solutions prepared in distilled water were used. Eggs of *Fundulus heteroclitus* were used in most experiments, although eggs of *F. majalis*, used occasionally, gave similar results.

RESULTS

A. Effect of Sucrose on the Internal Hydrostatic Pressure

As in earlier experiments (4), an internal hydrostatic pressure gradually developed when the egg was activated by the puncture with which the measuring micropipette was introduced. In some of the graphs showing the effects of sucrose, only the stabilized portion of the time course of internal pressure is shown since the earlier portion has been discussed previously. The effect of sucrose is osmotic, and is, in general, quite similar to that exerted by ions, but differing in amplitude and duration. The differences, as will be shown

later, can be attributed to the relative readiness with which these substances penetrate the chorion. The latter is a porous structure, and contrary to Loeb's conclusions (8, 9), is freely permeable to both water and ions (5). Since the ionic compositions of sea water and perivitelline contents are essentially the same, the effects of test solutions of sucrose, which were made up in sea water, are due entirely to the sucrose present. Depending on the concentration of sucrose, the effects are varied. At a concentration of 0.01 M, corresponding to an osmotic pressure of 150 mm. Hg, sucrose exerts the least effect. In Fig.

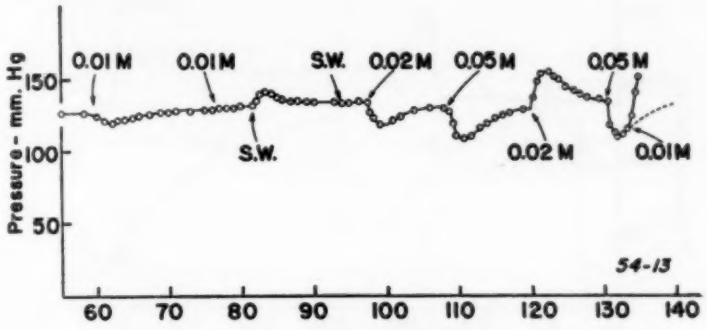

FIG. 1. Effect of hypertonic solutions of sucrose in sea water on the internal hydrostatic pressure. The first immersion in 0.01 M produced a slight decrease because this concentration was slightly hyperosmotic. The pressure returned when 0.01 M penetrated the chorion to establish a new equilibrium which was confirmed by the lack of effect of the second immersion in 0.01 M. The two subsequent immersions in sea water showed a similar process. 0.02 M sucrose when applied to an egg in sea water was hyperosmotic, but became hypo-osmotic if the egg had been first immersed in 0.05 M. At the end of the graph, perivitelline pressure probably would follow a slower return course as shown by the dotted line were the egg allowed to remain in 0.05 M sucrose in a manner similar to the results at 110 to 120 minutes. But changing to 0.01 M sucrose facilitated the pressure return.

1, the small transient decrease of pressure produced by this concentration can be explained by the fact that the perivitelline pressure was not quite 150 mm. Hg, and therefore, 0.01 M sucrose was slightly hyperosmotic. As when immersed in hypertonic salt solutions, water would leave the perivitelline space, accounting for the decline of pressure which would return when sucrose has penetrated the chorion to establish a new equilibrium.

Contrary to this simple manifestation, a 0.1 M sucrose solution in distilled water added to the sea water environment to yield a final concentration of 60 per cent sea water and 0.04 M sucrose produced a complicated sequence of events (Fig. 2). Immediately after the addition, there was a rapid rise in

pressure which was followed by a fairly rapid decline. This decline differed from that observed in hypotonic salt solutions (5) in being more rapid and in undershooting the original pressure to produce a dip before the pressure finally returned. This sequence of events can be explained as being the net result of two oppositely directed processes, one produced by the dilution of sea water, and the other produced by sucrose solution. The dilution of sea water (equivalent to 0.53 M of a uni-univalent salt) to 60 per cent (equivalent

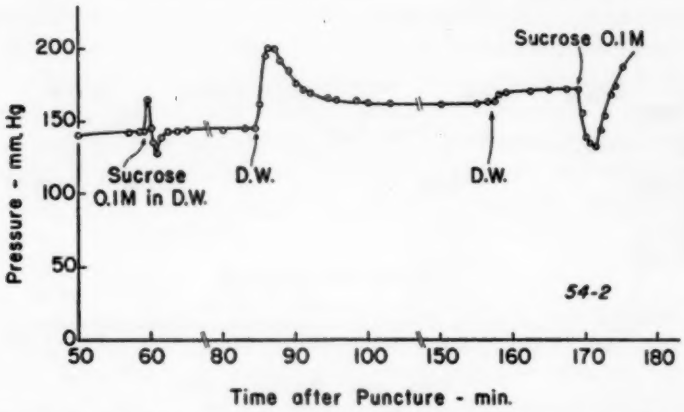

FIG. 2. Effect of 0.1 M sucrose in distilled water on the internal hydrostatic pressure. The first immersion produced a final concentration of 60 per cent sea water and 0.04 M sucrose. As a result, a diphasic response was elicited. That was due to salt dilution (causing pressure rise) and hyperosmoticity of sucrose (causing pressure decrease). The diphasic response was abolished after the salts were washed out by distilled water. The first wash at 82 minutes produced a large increase of pressure which then returned. At 120 minutes (not shown) a second wash produced a smaller rise. At 156 minutes, a third wash produced a very small change. After these washings, addition of 0.1 M sucrose in distilled water only produced the effect of hyperosmotic sucrose solution.

to 0.32 M) provided an osmotic gradient for water to enter the perivitelline space. The presence of 0.04 M sucrose on the outside, however, exerted the opposite effect since the perivitelline colloid was 0.01 M. The first process obviously exceeded the second in magnitude to produce an initial rise of pressure. The second process must not only have prevailed during this time to account for the rapid decline of the pressure, but also it must have outlasted the first to produce the transient dip in pressure. Evidence in support of this explanation is provided by the fact that the same concentration of sucrose when added to the same egg after the latter had been washed several times in distilled water produced only a marked decrease in pressure (Fig. 2). Un-

doubtedly, most of the salts in the perivitelline space had been washed out by the distilled water, so that the initial effect of salt dilution was no longer present.

In higher concentrations of sucrose, from 0.3 M to 1.9 M in distilled water or in sea water, the pressure was reduced to the atmospheric level or a slightly negative value for a varying period of time before the pressure finally began

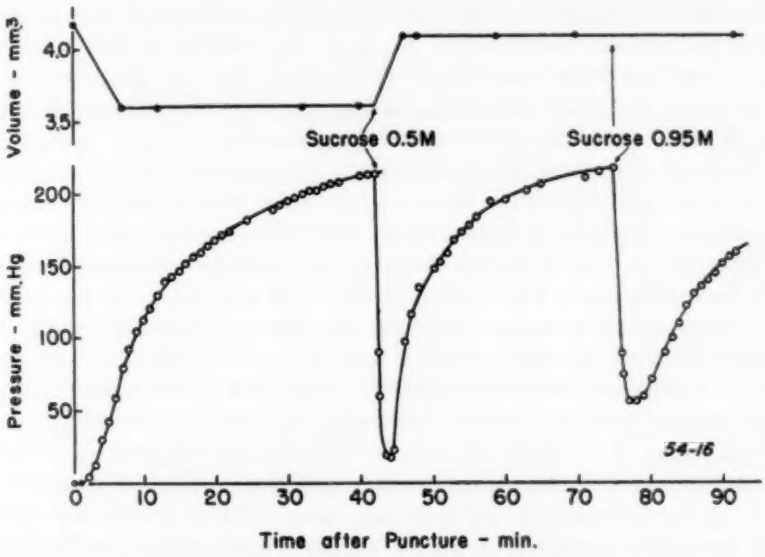

FIG. 3. Effect of highly hypertonic solutions of sucrose in sea water on the perivitelline pressure and the volume of the egg proper. In this egg, the pressure (lower tracing) was higher than average in the fully activated state, at which time the volume (lower tracing) was stabilized at a minimum. Addition of 0.5 M sucrose in sea water induced a precipitous fall of the perivitelline pressure and a simultaneous swelling of the egg proper. Upon the return of pressure, most eggs so treated showed a decrease of volume. In this case, no shrinkage was measured, suggesting that the egg proper had become incompressible at this time. Later immersion in 0.95 M sucrose caused only a pressure change without any further volume change since no previous shrinkage occurred.

to return (Fig. 3). This effect was also observed when mannitol was used instead of sucrose, showing that the effect was due only to the slowness with which the non-electrolytes crossed the chorion, and not to any specific action of sucrose. When the higher concentrations were used, the chorion often collapsed, producing a large indentation which then pressed on the egg proper. With time, the indentation in the chorion gradually refilled. But as long as a visible deformity was present, no pressure was recorded, the latter beginning

to return only after the last trace of the indentation was refilled frequently with some abruptness.

In all concentrations of sucrose, the pressure eventually returned, a fact which could only be explained by the penetration of sucrose through the chorion.

B. Further Evidence of Sucrose Penetration through the Chorion

The following experiment provides conclusive evidence of sucrose penetration through the chorion (Fig. 1). After first washing in 0.01 M sucrose solution and sea water successively to ascertain that the recording system was in order, sea water surrounding an egg with a stabilized pressure of 130 mm. Hg was replaced by a 0.02 M sucrose solution in sea water. A slight dip was produced since the applied solution was hyperosmotic. When the pressure had returned, a 0.05 M sucrose solution in sea water, was placed on the outside. This solution produced a more marked and more prolonged dip. While the egg remained in 0.05 M sucrose solution, the pressure eventually returned again, indicating that a new equilibrium had been reached across the chorion. Now, when a 0.02 M sucrose solution, the same concentration which previously elicited a decrease of pressure, replaced the 0.05 M solution, a transient hump was produced, indicating that 0.02 M was now hypo-osmotic. The stabilized internal pressure, however, has hardly changed from the original level. The occurrence of the hump can only be explained by the presence of 0.05 M sucrose in the perivitelline space to cause a 0.02 M solution to change from hyper- to hypo-osmoticity. Subsequently, when 0.01 M sucrose was substituted for 0.05 M sucrose (Fig. 1), the pressure, which probably would have followed the dotted line in its return, rose more rapidly and, in fact, exceeded the original level. This rapid return and increased pressure must also indicate the presence of 0.05 M sucrose in the perivitelline space, which transitorily was hyperosmotic to 0.01 M sucrose in the bathing fluid.

C. Volume Changes of the Egg Proper Accompanying Pressure Changes

As noted previously (4), after the chorion became inelastic, most of the perivitelline space which appeared was due to a decrease in the volume of the egg proper. This decrease in volume may vary between 20 and 40 per cent; the lower value being shown in the last column of Table I of the present paper. The variation is probably random among different batches of eggs, and does not affect the interpretation of the results of the experiment. In the last column of Table I are shown some values of the volume of the egg proper as a fraction of the whole. For each experiment, three values are shown, the first being that shortly after activation had begun and when some perivitelline space was present. The second value represents the fractional volume after or close to the attainment of a steady state following activation. The last value for each experiment is the fractional volume of the egg proper in

hypertonic sucrose solution obtained when the pressure was at its lowest for the test condition. As indicated in an earlier section, higher concentrations of sucrose often caused a collapse in the chorion which then pressed on the egg proper and induced deformation. For an accurate evaluation of volume from diameter measurement, the egg proper must be spherical, and distortions avoided. Therefore, Table I contains only those values which were not subjected to this complication. From the data, it is clear that in each case of immersion in a hypertonic sucrose solution that results in a significant

TABLE I

Volume of Egg Proper in Sea Water and in Sucrose

Experiment No.	Pressure	Medium	Volume of chorion	Volume of egg	Vol. egg / Vol. chorion
	mm. Hg		*mm.*^{3}	*mm.*^{3}	
54-6	758	Sea water	2.98	2.43	0.814
	902	" "	2.98	2.34	0.782
	755	Sucrose (1.9 M)	2.98	2.80	0.935
54-8	745	Sea water	3.18	2.84	0.894
	854	" "	3.32	2.68	0.808
	756	Sucrose (0.95 M)	3.32	3.02	0.910
54-16	750	Sea water	4.28	4.19	0.978
	957	" "	4.28	3.52	0.823
	895	Sucrose (0.5 M)	4.28	4.19	0.978
52-224	764	Sea water	3.59	2.95	0.821
	875	" "	3.59	2.67	0.744
	725	Sucrose (1.6 M)	3.59	3.26	0.907
54-15	761	Sea water	7.95	7.55	0.949
	918	" "	8.75	7.06	0.806
	755	Sucrose (0.5 M)	8.75	8.35	0.955

decrease of pressure, the egg proper enlarges. The swelling is prominent, nearly, but never completely obliterating the perivitelline space. Swelling occurred in all the eggs studied, and also in those eggs used for staining and development experiments (Section *E*) in which no pressure determinations were made. After a varying period of time, the egg proper usually shrank again. This secondary decrease in volume was always associated with an increase in the perivitelline pressure, the processes resembling those occurring during the early stages of activation (4).

D. Inverse Relationship between Pressure and Volume

In order to establish the dependence of the volume of the egg proper upon the perivitelline pressure, evaluation of PV is carried out. P is the absolute

TABLE II

Pressure-Volume Relation of the Egg Proper

Experiment No. medium	Chorion volume	Pressure	$\frac{P}{P_0}$	Egg volume	$\frac{V}{V_0}$	Relative PV
	mm^3	*mm. Hg*		mm^3		
54-6 (sea water)	2.98*	758	1.000	2.43	1.000	1.000
	2.98	777	1.025	2.34	0.963	0.987
	2.98	804	1.061	2.34	0.963	1.021
	2.98	830	1.095	2.34	0.963	1.054
	2.98	852	1.124	2.34	0.963	1.082
	2.98	870	1.148	2.34	0.963	1.105
	2.98	880	1.161	2.34	0.963	1.118
	2.98	888	1.172	2.34	0.963	1.128
	2.98	902	1.190	2.34	0.963	1.146
(sucrose 1.9 M)	2.98	755	0.996	2.80	1.152	1.148
	2.98	756	0.997	2.80	1.152	1.149
	2.98	771	1.017	2.68	1.103	1.122
	2.98	839	1.107	2.58	1.062	1.175
	2.98	866	1.142	2.52	1.037	1.184
	2.98	875	1.154	2.52	1.037	1.197
	2.98	901	1.189	2.52	1.037	1.233
54-8 (sea water)	3.18	745	0.971	2.84	1.014	0.985
	3.18	754	0.983	2.84	1.014	0.997
	3.32*	767	1.000	2.80	1.000	1.000
	3.32	805	1.049	2.68	0.957	1.005
	3.32	854	1.113	2.68	0.957	1.066
(sucrose 0.95 M)	3.32	756	0.986	3.02	1.079	1.063
	3.32	760	0.991	2.98	1.064	1.055
	3.32	799	1.042	2.92	1.043	1.086
	3.32	838	1.093	2.92	1.043	1.139
	3.32	868	1.132	2.92	1.043	1.180
	3.32	900	1.173	2.92	1.043	1.224
54-9 (sea water)	3.32	750	0.960	3.12	1.102	1.059
	3.45	767	0.982	2.94	1.039	1.020
	3.65*	781	1.000	2.83	1.000	1.000
	3.65	789	1.010	2.83	1.000	1.010
	3.65	800	1.024	2.83	1.000	1.024
	3.65	803	1.028	2.83	1.000	1.028
	3.65	825	1.056	2.83	1.000	1.056
	3.65	836	1.070	2.83	1.000	1.070
	3.65	847	1.085	2.83	1.000	1.085
	3.65	860	1.101	2.83	1.000	1.101
	3.65	863	1.105	2.83	1.000	1.105

TABLE II—*Concluded*

Experiment No. medium	Chorion volume	Pressure	$\frac{P}{P_0}$	Egg volume	$\frac{V}{V_0}$	Relative PV
	mm^3	*mm. Hg.*		mm^3		
54-10 (sea water)	3.69*	740	1.000	3.30	1.000	1.000
	3.69	758	1.024	3.14	0.952	0.975
	3.69	794	1.073	3.02	0.915	0.982
	3.69	834	1.127	3.02	0.915	1.031
	3.69	850	1.149	2.98	0.903	1.037
	3.69	870	1.176	2.98	0.903	1.062
	3.69	880	1.189	2.92	0.885	1.052
	3.69	895	1.209	2.90	0.879	1.063
54-16 (sea water)	4.28*	750	1.000	4.19	1.000	1.000
	4.28	841	1.121	3.59	0.856	0.961
	4.28	889	1.185	3.59	0.856	1.016
	4.28	957	1.276	3.52	0.840	1.072
(sucrose 0.5 M)	4.28	895	1.193	4.19	1.000	1.193
	4.28	955	1.273	4.19	1.000	1.273
52-224 (sea water)	3.59*	764	1.000	2.95	1.000	1.000
	3.59	797	1.043	2.95	1.000	1.043
	3.59	806	1.055	2.89	0.980	1.034
	3.59	846	1.107	2.89	0.980	1.085
	3.59	875	1.145	2.67	0.905	1.121
(sucrose 1.6 M)	3.59	735	0.962	3.26	1.105	1.063

pressure, and is the sum of the atmospheric pressure and the internal hydrostatic pressure. It prevails throughout the entire content held within the inelastic chorion, although a significant portion of it originated in the perivitelline colloid. In such a system, the volume of the egg proper V, if it is dependent on the perivitelline pressure, should vary approximately inversely as the pressure. At present, this relation can only be tested by evaluating PV during the normal course of increasing pressure, and, on occasion, when the perivitelline pressure was lowered by osmotic means. Since changes of osmotic gradients may produce effects of their own, the evidence would be stronger if the perivitelline pressure could be influenced independently by other means. Although no such method is yet available, at least one can be readily excluded. That involves changing the atmospheric pressure surrounding the whole egg, a procedure which will not materially occasion any transient pressure gradients between the egg proper and its immediate environment, the perivitelline contents, which retains its colloid osmotic pressure.

The results of the PV evaluation of 6 experiments are shown in Table II.

The criterion of using for volume determinations only those eggs which were spherical imposed a severe restriction on the amount of available data, because the period which these evaluations covered corresponded closely to the time of appearance of the blastodisc. The latter appeared first as a conical elevation and then as a discoidal collection at one pole of the egg proper which itself lost the spherical configuration. The values of P and V occurring when the chorion has become inelastic as indicated by the cessation of volume changes, have been taken as the basis of normalization. They have been desig-

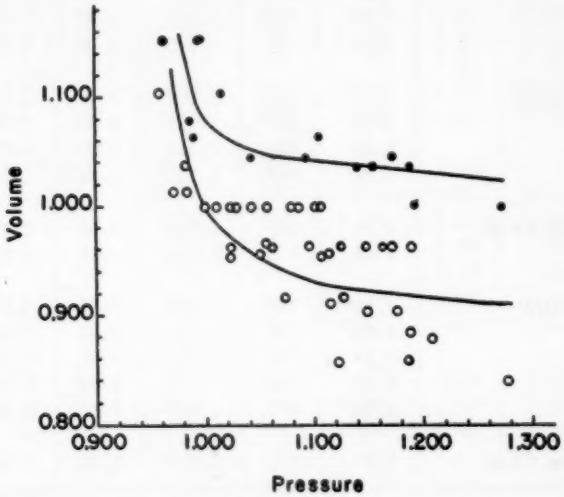

FIG. 4. Pressure-volume relation of the egg proper in sea water (hollow circles) and in sucrose (solid circles). Data are listed in Table II. Curves are traced by eye. In both media, the relationship is approximately a rectangular hyperbole. In sucrose, the curve is displaced upwards, indicating an increase of the incompressible fraction.

nated as P_0 and V_0 and have been assigned values of unity. In Table II, these values correspond to the row marked by an asterisk. All other values of P and V are fractions thereof (P/P_0, V/V_0); the column "relative PV" is the ratio PV/P_0V_0. Probably all the changes which apparently indicate some elasticity of the chorion can be attributed to experimental error in determining the diameter of the egg. In the experiments, the diameter and radii could be determined to 0.05 mm., approximately 3 per cent of the average diameter of 1.7 mm. Such an error in either direction will affect the volume by about 10 per cent, and is undoubtedly an important source of error.

In spite of this and other reasons to be discussed, it is evident from Table II that PV is relatively constant. The mean deviation is 8.53 per cent under various extreme conditions which are present or can be induced in the *Fundulus* egg. In Fig. 4 the pressure-volume relation of the data is shown. Clearly,

it has the shape of a rectangular hyperbola. The tendency towards attaining an asymptote along the pressure axis rather rapidly can be explained by the incompressibility of the egg interior. This factor, which is chiefly responsible for the deviation from ideality, is also evident from Table II. In it, larger deviations are seen to occur more often in the later stages of activation when the volume change has become limited. Fig. 5 illustrates the variation of PV as a function of P. The hollow circles indicate the relation in sea water. For an ideal gas there would be no deviation, and a horizontal line passing through

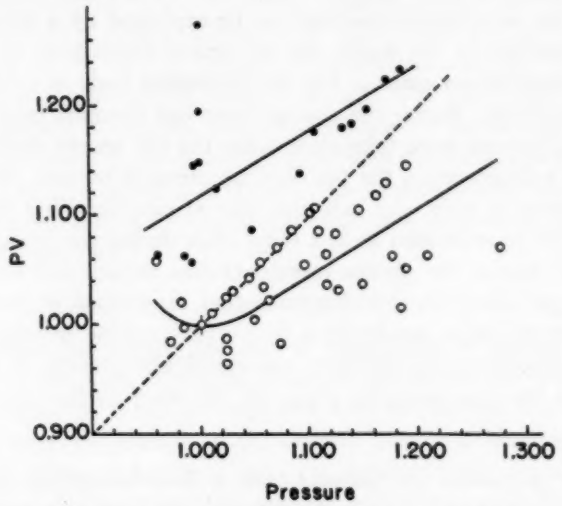

FIG. 5. Relation of PV to pressure. Hollow circles for values in sea water; solid circles, in sucrose. The dotted line has a slope of 1 and depicts the PV-P relation of an incompressible body. All values in sea water pass through the point of intersection of the normalized unity. The relation in sucrose is displaced upwards.

the point of intersection of unity would be present. For a completely incompressible body a line with a slope of 1 and passing through the same point would be the case, as indicated by the dotted line in the graph. The results of the measurement on *Fundulus* eggs fall somewhere in between the two limits, and must indicate that the egg proper does not behave as an incompressible body. At the pressures prevailing, this fact further illustrates that the plasma membrane is not always impermeable to water, because some egg content must be lost in order to account for the apparent compressibility (4). The incompressible fraction is analogous to the osmotically inactive fraction of the *Arbacia* egg (10) which must be subtracted from the volume in order for the PV relation of that cell to behave according to the Boyle, and van't Hoff laws.

After the egg had swollen in hypertonic sucrose and when sucrose penetrates

the chorion, an interesting shift of the *PV* curve occurs (Fig. 4). Satisfactory data for these conditions, free of all complications, are difficult to secure. But in all 3 sets of data obtained, there is a displacement of the *PV* curve upwards, indicating a larger volume in sucrose than in sea water at corresponding pressures. In other words, shrinking of the egg proper when immersed in hypertonic sucrose solution differs quantitatively from the change occurring in sea water in having a larger incompressible fraction. This change is also shown in Fig. 5, in which the *PV-P* relationship in sucrose is far removed from the normalized reference. The difference of the pressure-volume relationships in sea water and in sucrose can be explained by a difference in the stages of activation. In sea water, the egg proper starts from an unactivated state and changes into a more or less incompressible body of reduced volume. As stated previously, during the change some egg contents might have been lost. A similar process must take place when the egg proper shrinks in hypertonic sucrose solution when the perivitelline pressure returns. But the larger volume occurring in such eggs indicates that at corresponding pressures, less egg content can be expressed at this stage than during the process of activation. In other words, the plasma membrane has become less permeant as a result of changes taking place during activation. Hypertonic sucrose solution in the external environment, producing a drop in perivitelline pressure, apparently increases the membrane permeability non-specifically. Results in the following section and in the accompanying paper (3) will show further evidence of this effect.

E. Effect of Sucrose on Staining with a Non-Penetrating Dye, and on Development

To test the possibility that the effect of sucrose is to produce a non-specific increase of the membrane permeability, a solution of brom-creosol purple was used as an indicator. A batch of 20 fertilized eggs having well formed perivitelline spaces was immersed in sea water containing 0.04 per cent brom-creosol purple. Another batch was placed in a solution of 50 per cent sea water and 0.96 M sucrose, containing the same concentration of dye. The indicator was purple in both solutions because of the alkaline sea water, but changed color to greenish yellow at pH 6.8. Being a sulfonated dye, it normally does not enter a cell from without. After immersion for 12 hours in each of the solutions, the eggs were washed in a large volume of sea water to eliminate the purple colored dye in the perivitelline space. Eggs from each solution were then examined against a white background of a thin layer of transilluminated paraffin wax. It could be easily determined that the eggs soaked in sea water and indicator alone were not dyed because the yolk retained its original color, while the eggs immersed in the solution with a hypertonic concentration of sucrose possessed a greenish yellow yolk sac. The difference

in color was especially prominent when an egg from each solution was placed side by side and both examined within the same field with a dissecting microscope.

If a hypertonic sucrose solution does alter the characteristic of the surface of the egg proper, the extent of its deleterious effects must be estimated. For this purpose, a batch of eggs was fertilized in sea water. When the perivitelline space was well formed, 60 of them were transferred to 0.95 M sucrose in sea water in which the perivitelline space all visibly decreased, and the chorion collapsed. After half an hour, the period usually allowed in the pressure experiments, half of the eggs in sucrose were transferred back to sea water, and the other half left to develop in hypertonic sucrose solution. Of the 30 eggs which underwent transient immersion in sucrose 29 of them hatched. The same result was observed in another 30 untreated ones. Of the 30 eggs remaining in sucrose, none of them hatched, and most of them died after the heart beat was present. Two striking features of the embryos in sucrose were the small size of their yolk sacs and the markedly sludged blood flow, both being consistent with a state of dehydration.

DISCUSSION

From a continuous record, the rate of penetration of sucrose through the chorion can be obtained. It can be assumed that no sucrose is present in the perivitelline space in the beginning, and that the rate of the return of the pressure is dependent directly on the amount of sucrose entering through the chorion. From these, the rate of entry can be determined by the rate of the pressure return. For instance, for one egg in 0.5 M sucrose, 7 minutes were required for half of the original pressure and 18 minutes for all of the pressure to return. Such evaluation, however, was soon abandoned because much variation was present. Thus, with any one concentration, different eggs had quite different rates; similarly, for any one egg, different concentrations did not produce any orderly sequence of rates of penetration. Variations like these must therefore indicate that the chorion is a rather heterogeneous structure with varying numbers of pores of different sizes.

The dependence of the volume of the egg proper upon the perivitelline pressure is clearly shown in the results. Further support is provided by the fact that an inverse relation between pressure and volume occurs in both sea water and sucrose, and at different stages of activation. Better agreement with ideality may be obtained if a correction is made for the incompressible fraction. Such a correction has been avoided, however, because unlike the case of the *Arbacia* egg (10), the permeance properties of the *Fundulus* egg change with time. Without more knowledge of the time course of this change, such corrections appear superfluous in the presence of other experimental errors.

Part of the mechanism responsible for a change of permeance properties in the *Fundulus* egg may be examined by contrasting the various phenomena of activation with those of the lifting of the fertilization membrane in some echinoderm eggs. For the *Fundulus* egg, both the volume and surface area decrease during activation when the perivitelline colloid swells, separating the egg proper from the inelastic chorion. Recent experiments (11) suggest that the lifting of fertilization membrane is also attributable to imbibition of a released colloidal substance external to the ovum. The fertilization membrane, however, is not rigid; swelling of the colloidal material causes it to expand without affecting the volume of the egg proper. This constancy of the volume and surface area of the ovum, and a finding that water permeability increases on fertilization (7) serve as illuminating contrasts to the changes in the *Fundulus* egg. In addition, recent experiments have shown that the ionic permeability of the *Fundulus* egg decreases about fivefold (2, 3) upon activation, while in the starfish egg no significant difference occurs before and after fertilization (14). Thus, the available evidence, direct as well as contrasting, supports the suggestion that the acquisition of the impermeable properties of the *Fundulus* egg during activation is at least in part related to a decrease of the surface area. Were the decrease of surface area to affect mainly the diameter of the pores of the plasma membrane then the change in permeance properties is not surprising (*cf.* reference 4). Probably such a mechanism also exists in the trout and the salmon eggs, and perhaps in other teleost eggs as well.

However, some other changes, the nature of which is unclear, must function in conjunction with this mechanism. That the perivitelline pressure does not account for all the changes of permeance is clearly shown by the shift of the pressure-volume relation in sucrose and late in activation. If only the size of the pores were involved, then the pressure-volume relation in sea water during activation and in sucrose late in activation would be superimposable. But the presence of a larger incompressible fraction late in activation must reflect a more lasting alteration of the plasma membrane as a result of activation. The volume changes observed are undoubtedly accomplished by water filtration through pores. A more or less permanent narrowing of the pores following activation may account for the shift of pressure-volume relation. However, these interpretations still cannot explain the absence of D_2O diffusion across the trout egg (6, 13) and the salmon egg (11). On the other hand, the suggestion that these plasma membranes are composed of a complete lipide phase (13, 15) does not agree with the values of membrane capacity (3).

SUMMARY

Upon activation, an internal hydrostatic pressure develops within the *Fundulus* egg, and compresses the egg proper to a reduced volume. When the

perivitelline pressure is abolished by a highly hypertonic sucrose solution, the egg volume increases. As sucrose penetrates the chorion, the volume again decreases. The relation between P and V in these conditions is inverse, and approximates a rectangular hyperbola. The limiting factor causing most of the deviation is shown to be the incompressible fraction. It is concluded that the volume of the egg proper is controlled by the perivitelline pressure, and that the effect of hypertonic sucrose solution is exerted by lowering the pressure and thereby increasing membrane permeability non-specifically. It is also shown that some permanent alterations occur within the plasma membrane during activation that reduce the permeance, and thereby, increase the incompressible fraction.

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CHANGING ELECTRICAL CONSTANTS OF THE FUNDULUS EGG PLASMA MEMBRANE*

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(Received for publication, March 7, 1956)

INTRODUCTION

Much valuable knowledge concerning the nature of the plasma membrane has been obtained by combining information acquired from kinetic permeability studies with that resulting from various physical measurements (*cf.* reference 7). In general, it is now believed that the plasma membrane is a mosaic structure composed of lipides and proteins with interposing pores throughout (*cf.* reference 18). Electrically, the plasma membrane resembles a resistive-capacitative circuit, a fact which itself indicates the mosaic nature of its structure. By its very nature, the capacitative component is considered to be the ion-impermeable elements (3). Data obtained from a variety of cells (4) indicate that most cells have a membrane capacity of *circa* 1 μF/cm.^{2}, suggesting that the ion-impermeable portions probably are due to geometrically prominent fractions of the plasma membrane with relatively similar thicknesses and compositions. On the other hand, the resistive component varies markedly among different cell types, and analogous cells of different species. In contradistinction to the capacitative elements, the resistive component has been thought to be due to small but crucial sites for the penetration of charged particles.

In the eggs of some teleosts, a profound change in the permeance characteristics of the plasma membrane occurs during activation (*cf.* reference 12). In the fully activated stage, these eggs are impermeable to water and ions. On the basis of an absence of D_2O exchange, Ussing (25) suggested that the fertilized trout egg had a plasma membrane which was composed of a continuous lipide phase without any pores. This suggestion would require the

* This work was carried out at Woods Hole in a laboratory provided for Dr. H. Grundfest (Department of Neurology, Columbia University, College of Physicians and Surgeons) by a grant from the Marine Biological Laboratory, under its ONR contract (Nonr-09703). I am indebted to Dr. Grundfest for all the facilities placed at my disposal. To the Director and staff of Marine Biological Laboratory, I am grateful for the generous assistances.

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accompaniment of some other unusual properties which can be detected by measuring the electrical constants of the plasma membrane. Previously, Rothschild (20) had shown that the membrane capacity of the trout egg was 0.58 μF/cm.^{2}, a value rather close to that of other cells which were known to be permeable to water and ions.

The purpose of this paper is to present the results of some experiments with the *Fundulus* egg toward this end. The electrical constants have been measured and their significances are discussed. Furthermore, sequential measurements during the activation process contribute some information with regard to the alterations within the plasma membrane, which are perhaps responsible for its ultimate impermeability.

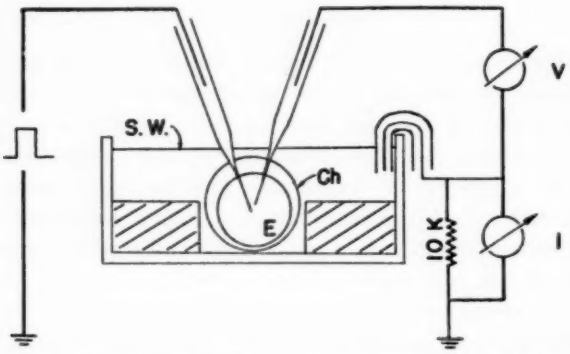

FIG. 1. Scheme of experimental arrangement. The egg is held in a shallow hole cut in some paraffin wax in the bottom of a dish. The outer shell is the non-elastic chorion (*Ch*). The microelectrodes are in the egg proper (*E*). *V* is for recording potential; *I*, current. The whole egg is covered by sea water (S.W.) or sucrose in sea water.

Material and Method

Mature eggs stripped from female *Fundulus heteroclitus* or on rare occasions from *F. majalis* were used in the experiments. No apparent difference was noted for the two species. The general experimental arrangement and technique were similar to those employed for inserting a micropipette into the interior of the egg for measuring hydrostatic pressure (13). Individual eggs were held in a small depression in some paraffin wax, and were impaled with two microelectrodes from the top at a slight angle (Fig. 1). The microelectrodes (16), filled with 3 M KCl (17) were made by a method of prefilling (11). One of the electrodes was used for passing a square pulse which could be varied in amplitude and duration. The other electrode was used for recording the membrane potential changes produced by such a pulse. It was connected to a high impedance, negative capacity input amplifier[1]. The indifferent elec-

[1] Designed by Mr. E. Amatinek (Department of Neurology, Columbia University, College of Physicians and Surgeons) whose expert assistance also facilitated the progress of this work.

trode in the bath was connected to a 10,000 ohm resistor, the other end of which was grounded. Voltage was recorded differentially between the microelectrode and the indifferent electrode. The current recording amplifier was connected across the resistor.

RESULTS

A. Membrane Potential.—With a capillary electrode of 70 μ diameter in the perivitelline space and another in the surrounding fluid, Sumwalt (21) had measured a potential difference of several millivolts across the chorion, when the external fluid had a concentration of K^+ close to that of sea water. In the presence of a non-diffusible colloid which may be charged, a Donnan potential may well be expected. The perivitelline colloid may be amphoteric, and its charge subject to change depending on the pH of the medium. This factor may be contributory to the reversal of polarity of the potential as observed by Sumwalt (22) to occur at pH 3.5, in addition to an alteration of the charges of the chorionic components. These observations, while not pertaining to the plasma membrane, are interesting because they show that the chorion, a non-viable structure, resembles a colloidion membrane with large pores, a conclusion which has also been reached *via* osmotic studies (14).

In the present experiments, the microelectrodes were inserted deep into the interior of the egg proper. The current and the potential were passed and measured across the plasma membrane and the chorion. The microelectrodes were always introduced into the unactivated egg which had a rather soft chorion and no perivitelline space. Throughout the experiments, no consistent membrane potential was observed. There were occasional changes of a few millivolts which could not be distinguished from experimental error. When activation proceeded and a distinct perivitelline space was formed, there were still no appreciable changes in membrane potential. The apparent absence of a membrane potential raises some interesting questions which must remain in abeyance until the cationic content of the interior of the *Fundulus* egg is known. As will be shown in the next section, real entry into the cell has been achieved, and the absence of a membrane potential cannot be attributed to non-penetration as has occurred with earlier reports on the echinoderm eggs (*cf.* reference 24). Neither can the absence be due to short-circuiting paths around the shaft of the electrode because of the high membrane resistance measured.

B. Membrane Resistance and Capacitance.—Penetration of the microelectrode through the plasma membrane could be observed visually. To verify this fact, the recorded form of the current pulse was invaluable especially since no distinct change in potential could be used as an index of entry into the cell proper. Fig. 2 shows the current (lower) and voltage (upper) traces as recorded on the oscilloscope. In *A*, both the polarizing and recording microelectrodes were in sea water which bathed the indifferent electrode. The cur-

rent trace recorded a long square pulse of 70 msec. duration. Except for two short lived capacitative surges at the make and the break of the pulse, the voltage line was undisturbed. In *B*, both microelectrodes have been inserted into the center of an egg proper. Under this condition, the current path included the resistance of the plasma membrane and the chorion. As is to be expected, there was a large IR drop observed on the voltage trace. Owing to the capacity of the plasma membrane, the voltage transients also exhibited

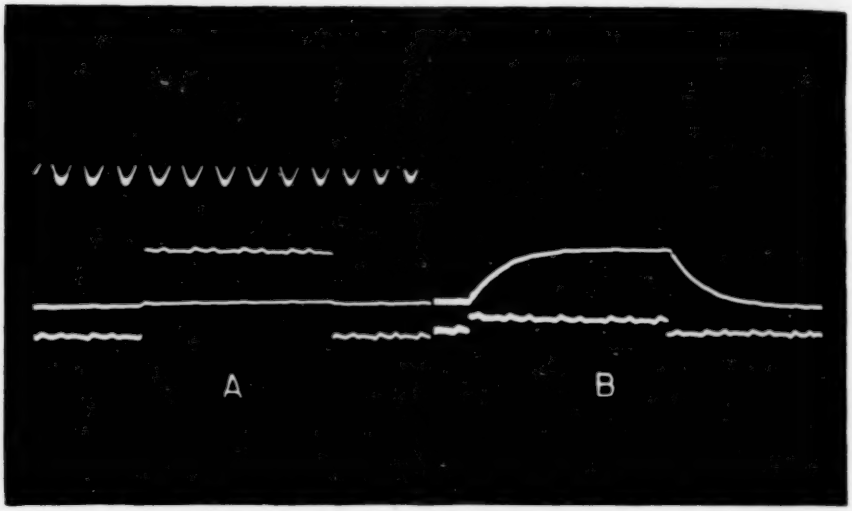

FIG. 2. Current and voltage traces before (*A*) and after (*B*) penetration of microelectrodes into the egg proper. Upper trace, voltage; lower trace, current. Amplification for current trace is higher, accounting for the noise present. Time 100 cycles/sec. The current strengths are not the same. Before entry, current pulse, even when strong, does not produce any significant change in the voltage line. After entry, small current pulses produce marked voltage changes which also show the exponential rise and decay.

the characteristic exponential rise and decay. Almost all these features were due to the electrical properties of the plasma membrane because the contributions of the chorion, the yolk and cytoplasm, and the perivitelline contents were negligible. This was evidenced by the fact that after the entire egg proper was disrupted by mechanically stirring with the microelectrodes, the current and voltage traces reverted back to the forms observed when the microelectrodes were in sea water.

As shown in Fig. 3, D.C. resistance is determined from the slope of current-voltage relationship dV/dI. To avoid possible polarization of the current microelectrode, inward (anodal) and outward (cathodal) pulses were alter-

nated during the experiment for each current strength used. The oscilloscopic traces were photographed and the records enlarged approximately 10 times for examination. The resistance so obtained is the total resistance (r_m). In order to compare the state in the unactivated eggs with that of the activated eggs which differ in volume and surface area, r_m is converted to R_m which is

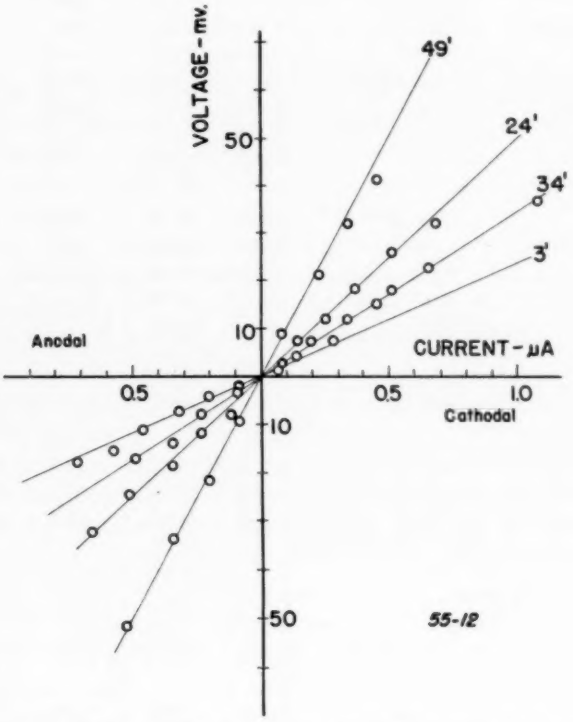

FIG. 3. Current-voltage relationship of an activating egg. At 3′, some platelets were still visible; the egg was considered unactivated. At 34′, current-voltage relation was determined within 1 minute after immersion in a hypertonic sucrose solution. Note that the slope decreased from that at 24′, but later again increased. The values for the various parameters are given in Table I.

membrane resistance in ohm-cm.^{2}, surface area having been obtained from diameter measurement. From the enlarged records, the time constant (τ) of the voltage transients, *i.e.* the time at which $1/e$ of the final stable value is attained, can be determined. From the time constant and R_m, membrane capacity C_m in μF/cm.^{2} is obtained from $\tau = RC$. Sometimes, the polarizing electrode was of high resistance, and a long pulse was needed to allow attainment of an ultimate steady state. In such cases, the sweep was necessarily

TABLE I

Electrical Constants of the Fundulus Egg Plasma Membrane

Time	Condition	Area	r_m	R_m	τ_m	C_m
min.		*cm.²*	*ohm*	*ohm-cm.²*	*msec.*	*μF./cm.²*
Exp. 55-10						
1½–2½	Unactivated	0.108	28200	3040		
26–27	Activated	0.104	57800	6010	2.48	0.41
48–49	"	0.099	57800	5720	2.48	0.43
53–54	0.25 M sucrose	0.108	65800	7100	2.48	0.35
66–67	0.25 M "	0.108	82300	8870	3.42	0.39
78–80	0.25 M "	0.108	82300	8870	3.60	0.40
85–87	0.38 M "	0.108	82300	8870	3.42	0.39
Exp. 55-11						
10–11½	Activated	0.096	73700	7090	5.0	0.71
34–36	"	0.091	108000	9850	4.70	0.48
44–45½	"	0.080	127000	10100	3.92	0.39
50½–53	0.25 M sucrose		40100		3.34	
94–96	0.25 M "		126000		3.72	
100½–102	0.38 M "		126000		5.40	
103–105	0.38 M "		94400		3.52	
121–123	0.38 M "		108000		3.81	
Exp. 55-12						
3–4	Unactivated	0.118	25200	2580	2.10	0.81
9–11	Activated	0.095	55200	5350	3.36	0.63
22–24	"	0.096	55100	5300	2.66	0.50
34–35	0.25 M sucrose		33700		0.97	
36	0.25 M "		38200		1.03	
42	0.25 M "	0.096	83000	7960	3.18	0.39
45½–46	0.38 M "	0.104	92300	9600	3.04	0.32
49	0.38 M "	0.107	92300	9880	4.28	0.43
Exp. 55-13						
1½–2½	Unactivated	0.116	15500	1860	4.75	2.56
9–10	Activated	0.078	158000	12300	10.00	0.82
30	"	0.086	158000	13600	5.33	0.39
Exp. 55-15						
3	Unactivated	0.116	35700	4140	2.49	0.60
9–10	Activated	0.095	71400	6790	4.18	0.62
21–24	"	0.096	93800	9000	5.66	0.63
49–50	"	0.093	132000	12300	7.09	0.58
Exp. 55-16						
3–4	Unactivated	0.111	51700	5750	10.00	1.74
20–23½	Activated	0.105	127000	13300	10.00	0.75
40–42	"	0.105	167000	17500	8.29	0.47
54–56	"	0.105	167000	17500	6.57	0.38
58–60	0.20 M sucrose	0.107	167000	17900	9.90	0.57
68–69	0.20 M "	0.105	192000	20200	9.45	0.57
89–90		0.105	238000	25000	10.06	0.43

reduced, a maneuver which affects the accuracy of the τ determination, and consequently C_m.

Unactivated Egg.—The microelectrodes were inserted into mature eggs within 1 to 2 minutes after the latter were stripped from female fish. Square pulses were repeated at 1/sec. while the electrodes were being inserted. Upon penetration, a sudden change in the voltage trace served as definite proof of entry. Current-voltage relationships were determined for different currents and were completed within another 1 to 2 minutes. Occurrence of activation was also verified by the persistence or absence of cortical platelets by direct observation. All the values in Table I for unactivated eggs are for those eggs in which the platelets were still present, at least in part, at the end of the first series of determinations. As shown, R_m for unactivated eggs range from 1860 to 5750 ohm-cm.^{2}, with a mean of 3450 ohm-cm.^{2}

Activated Egg.—As described previously (10, 13), puncture induces activation in the *Fundulus* egg, resulting in a sequence of changes which are indistinguishable from those initiated by successful insemination. Such activated eggs always reached the blastodisc stage, and sometimes might even undergo the first cleavage. Because of such similarity, the present results may perhaps be applied to the fertilized egg as well. The time resolution of the present experiments was limited by the necessity of alternating inward and outward current pulses; and a continuous time course could not be obtained. But it is clear that total resistance (r_m) began to increase almost immediately upon the complete disappearance of the platelets. Subsequent determinations carried out at intervals show a progressive increase of r_m which tended to reach some steady level at about an hour following the beginning of activation. Throughout this time the perivitelline space has also been enlarging, and, as has been shown, a hydrostatic pressure was developing (13). All these phenomena were accompanied by a reduction in the volume and surface area of the egg proper.

With a reduction in surface area, R_m, membrane resistance in ohm-cm.^{2}, has to be evaluated. From Table I, it can be seen that not only r_m, but also R_m increases as activation proceeds. In the ultimate steady state, R_m of activated eggs ranges from 8870 to 25,000 ohm-cm.^{2}, with a mean of 13,290 ohm-cm.^{2} Although a threefold range is present among these values, for any individual egg the change of membrane resistance upon activation is consistently in the increasing direction, the increase being 2 to 7 times that of the unactivated egg. Fig. 3 illustrates the current-voltage relationships of one egg at different times after activation, and shows the progressive increase of the slope of dV/dI.

In Figs. 4 and 5 are shown the relationship of surface area to r_m and to R_m respectively. Except for a few points in the right upper corner, all the others follow a distinct distribution which indicates an increased r_m or R_m with decreasing surface area. The aberrant points are all from one egg,

volume changes of which were small and subject to much inaccuracy of measurement. The points plotted include both the ones obtained during the activation process, and those present when the egg proper had been induced to

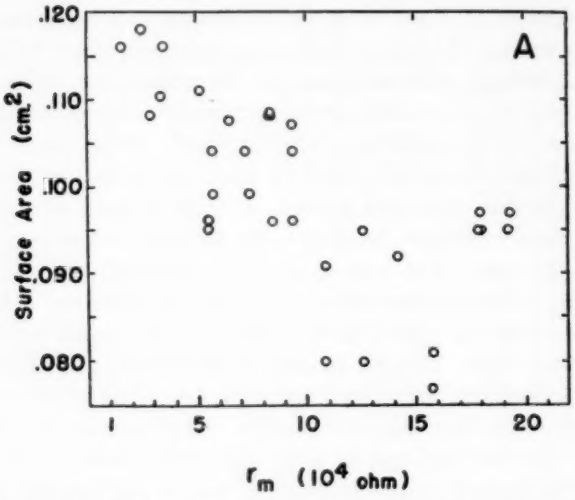

FIG. 4. Relation of surface area to r_m. The distribution indicates an increased r_m with decreased surface area.

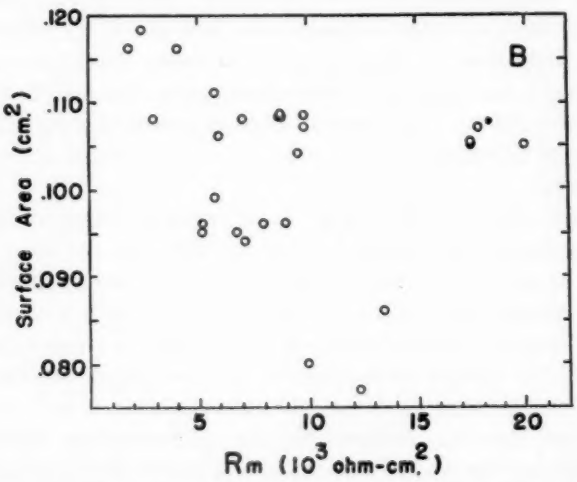

FIG. 5. Relation of surface area to R_m. See text for discussion.

swell by a hypertonic sucrose solution (*cf.* p. 115). If the increase of total resistance (r_m) is only caused by a reduction in the surface area through which the applied current is dissipated, membrane resistance evaluated on a basis

of equal area (R_m) should not be affected. Fig. 5 shows that increase of resistance is not attributable completely to a decrease of surface area. It further emphasizes that a more subtle and more permanent alteration takes place within the plasma membrane during activation (12).

Reversal of the Membrane Resistance Changes.—As shown in a previous paper (12) a hypertonic sucrose solution when added to the external environment releases the perivitelline pressure and causes the egg proper to swell, swelling being accompanied by an increase in the surface area. This phenomenon has been employed in the present experiments. During the increase of r_m and R_m accompanying the shrinkage of the egg proper, highly hypertonic solutions of sucrose were applied to the environment. The egg proper promptly enlarged. Within the first minute of swelling, both r_m and R_m dropped markedly, and approached those values present in the unactivated egg. They returned rapidly and usually high values were again attained before any significant secondary changes in the size of the egg proper could be detected. The effect is illustrated in Fig. 3 in which the line marked 34′ indicated the current-voltage relationship 1 minute after immersion in 0.25 M sucrose in sea water. r_m dropped from 5.51×10^4 ohms measured at 24 minutes after activation to 3.37×10^4 ohms upon immersion in sucrose. The rapid return of resistance is shown by the fact that at 36 minutes r_m is already 3.82×10^4 ohms, and at 42 minutes, r_m has attained 8.30×10^4 ohms. Such a rapid return of the membrane resistance accounts for the apparent absence of changes on some occasions in which the egg proper had visibly swelled. In all such cases, measurements could not be carried out within the first 2 minutes after immersion in sucrose.

Capacity.—The determination of membrane capacity in the present experiments is not of a high order of accuracy, since slight errors in estimating the time constant may seriously affect the capacity value. The average C_m for both unactivated and activated eggs is 0.62 μF/cm.^{2}, a value which corresponds well to the value for many other types of cells, *viz.*, 1 μF/cm.^{2} (4). From the best experiments in which the rise and decay portions of the voltage transients were expanded on a fast sweep, no significant alteration of C_m could be detected during the volume and surface area changes.

DISCUSSION

Based on the lack of D_2O exchange across the fertilized trout egg, Ussing (25) suggested that the plasma membrane of the trout egg is composed of a complete lipide phase with no pores. As a result, water molecules cannot traverse it either by solution in the lipide phase or by filtration through pores under osmotic or hydrostatic gradients. The similarity between the *Fundulus* and trout eggs with regard to their water and ion impermeability has been discussed previously (13, 14). Probably the structure of the plasma membrane of these two eggs is also similar. Although the absence of a membrane po-

tential in the *Fundulus* egg cannot as yet be fully accounted for, the acceptance of the above suggested nature of the plasma membrane casts some doubt on the hypothesis of phase-boundary potentials as the basis of membrane potentials in biological systems (1). In various excitable tissues (8) and in an echinoderm egg (24), the existence of a membrane potential is associated with a membrane resistance of *circa* 1000 ohm-cm.^{2}, suggesting that higher ionic fluxes must underlie the membrane potentials, although the exact mechanism is still unclear (6). This reasoning, however, cannot account for the absence of a membrane potential in the unactivated *Fundulus* egg.

As illustrated in Fig. 3, the current-voltage relationship of the *Fundulus* egg plasma membrane remains linear even up to current densities of 10 μA/cm.^{2} of inward as well as outward directions. With a membrane capacity of *circa* 0.5 μF/cm.^{2}, the charge displaced is about 5×10^{-8} coulombs/cm.^{2} at the highest current. This quantity is one order of magnitude larger than the threshold value for exciting the squid giant axon (8). A similar linear current-voltage relationship is also observed (24) for the starfish egg at even higher current densities. This linearity indicates clearly that two electrically inexcitable animal ova do not possess specialized cationic transport mechanisms as do nerves and muscles.

The hypothesis that a complete lipide phase accounts for the permeance peculiarities of these plasma membranes (25) however, appears to be at odds with the values of membrane capacity, 0.58 μF/cm.^{2}, and 0.63 μF/cm.^{2} for the trout and the *Fundulus* eggs respectively. Membrane capacity varies directly as the dielectric constant, an indication of the composition, and inversely as the thickness of the plasma membrane. Taking the lipide-protein ratio of 1:1.7 of the erythrocyte plasma membrane (18) as representative of cells with a membrane capacity of *circa* 1 μF/cm.^{2}, two alternative interpretations can be offered for the plasma membrane of the teleost eggs. First, it has a similar composition to that of erythrocytes and many other cells, in which case, the unique impermeable properties must be explained by lack of pores in the plasma membrane. Second, if a complete lipide phase prevails, the dielectric constant would be high, and could only be counterbalanced, for the membrane capacity, by an increase in the thickness of the plasma membrane, possibly to within the resolution of present experimental methods. Both alternatives can be experimentally investigated, but at present, the observations during the activation process favor the former.

As has been shown, during activation membrane resistance increases markedly while membrane capacity remains unchanged. Categorically, the alterations in the plasma membrane which lead to an increased electrical resistance may come about in two ways. It may be due to the formation of additional lipides in the plasma membrane, increasing the thickness, and further slowing the diffusion of molecules and ions through it. Or alternatively, it may be

due to a decrease in the effective pore size, with the tacit assumption that under a potential gradient most of the ionic movements occur within the continuous aqueous phase which is present in the pores and their channels. The first is made unlikely by the finding that the membrane capacity does not change significantly during activation, because a change in the lipide nature or content should be manifested in the capacity measurement. In addition, if no carrier systems are present within the membrane phase, a membrane of hydrophobic lipides would behave like parallel plate condensers, and would provide infinite D.C. resistance. In any event, it seems that diffusion of aqueous particles through a lipide phase should depend more on its solubility in the lipide than on the thickness of the latter. Constancy of membrane capacity was also observed by Rothschild (20) in unfertilized and fertilized trout eggs. The observation, however, was weakened because no mention was made of whether or not the unfertilized eggs were activated.

The second possibility of the fundamental changes in the plasma membrane during activation depends on the assumption that ionic movements occur partly through pores in the plasma membrane. No serious objections to this assumption can be raised since the ions are in an aqueous phase which is continuous through the channels provided by the pores. Despite the numerous carrier mechanisms postulated for active ion transport across membranes (*cf.* reference 2), it is implicitly agreed that aqueous diffusion plays an appreciable role in the passive transfer of ions across the plasma membrane (23, 25). The linearity of the current-voltage relationship indicates that ions can be moved across the plasma membrane of the *Fundulus* egg under a potential gradient of either polarity. Although it measures the resistance offered by the membrane components to movement of charged particles, the ohmic nature of the relationship does not favor the presence of any active transport. Thus, the ionic movement which occurred under the conditions of the experiment is most likely passive in nature. Another indication that some passive ionic movement occurs through pores is shown by the modifying effects produced upon it by concomitant water filtration; facilitated if water moves in the same direction as the ions, and retarded if water moves in the opposite direction. In the case of the isolated frog skin, Na^+ movement has been shown to occur in part through pores in the presence of neurohypophyseal extract which increases water filtration (15) and, simultaneously, Na^+ influx (26).

For the *Fundulus* egg, previous osmotic studies (12, 14) suggest that upon activation the effective pore size in the plasma membrane is reduced, the decrease accounting mainly for the water impermeability. This has been verified with D_2O exchange for the salmon egg (19). The present experiments further support this view. The lower membrane resistance values of the unactivated egg and eggs induced to swell by hypertonic sucrose solution are

accompanied by a larger surface area, which must in some way affect the pores available for aqueous exchange. In the eggs that were induced to swell, the rapid drop of membrane resistance is also consistent with the swelling of the egg proper which has been attributed to entry of water into the egg proper. The lower membrane resistance indicates that ionic movement through pores is 2 to 7 times easier than through the membrane phase itself which predominates in the activated egg. For the latter, absence of an active transport system does not exclude the operation of a thermodynamically reversible ferrying mechanism in the membrane phase such as that responsible for exchange diffusion (25).

Ionic movement through pores differs from water filtration *en masse* in that Poiseulle's law should not affect the former. The relationship between the electrical resistance and effective pore size, however, remains complex. Obviously, changes in the pore size cannot be obtained simply from changes in the total surface area. Electrical resistance, while providing some information concerning the sum total of the resistances of all the pores, does not indicate the number or the size of individual pores. The latter information can only be provided by kinetic permeability studies with substances of varying molecular volumes (*cf.* references 5 and 7). Nevertheless, it is clear from Table I that less than 20 per cent, or even as little as 5 per cent, of the surface area of plasma membrane is involved in the drastic changes of the permeance properties.

SUMMARY

Electrical constants of the plasma membrane of the *Fundulus* egg have been measured with microelectrodes by the transient method. No consistent and significant membrane potential was measured. Membrane capacity averages 0.63 μF/cm.^{2} for both unactivated and activated eggs. Membrane resistance averages 3450 ohm-cm.^{2} in the unactivated eggs, but increases 2 to 7 times to an average of 13,290 ohm-cm.^{2} in the fully activated state. In a hypertonic sucrose solution, the swelling of the egg proper is accompanied by a rapid fall of membrane resistance towards that in the unactivated state. The changes of the membrane resistance are interpreted as probably caused by alterations in the effective pore size in the plasma membrane.

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AN ADENOSINETRIPHOSPHATE-ACTIVATED HEMOLYTIC SYSTEM

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(Received for publication, March 8, 1956)

INTRODUCTION

In 1954 Synder and his coworkers (1) reported that water hemolysates of rabbit erythrocytes were capable of lysing whole rabbit cells if adenosinetriphosphate was also present in the system. Sheep cell hemolysate and adenosinetriphosphate did not have a corresponding effect on sheep erythrocytes. Subsequently, one of the group, (M.R.B.),[1] found that rabbit cell hemolysate also caused hemolysis of human erythrocytes in the presence of adenosinetriphosphate, whereas human hemolysate showed no lytic action on human cells under the same conditions. Since this system is unique in that a source of potential high energy phosphate appears to be involved in the hemolytic process, we thought it of interest to study it in some detail.

In this paper, studies on the activation and inhibition of the system and the preparation of a partially purified fraction obtained by fractional centrifugation of rabbit hemolysate are described. The concentration of adenosinetriphosphate is followed during the course of hemolysis and its possible role in the system is discussed.

Materials and Methods

Preparation of Diluents and Glassware.—Since the hemolytic system was found to be inhibited by distilled water as obtained from the usual metal still, all reagents were dissolved in water which had been passed through a Barnstead demineralizer and then distilled in an all-glass apparatus. All glassware was cleaned in sulfuric acid–dichromate solution and after preliminary rinsing in tap water was washed ten times with demineralized water and three times with glass-distilled water. When plastic tubes or dialysis casings were used they were soaked overnight in 0.1 M versene and then washed as above with demineralized and glass-distilled water.

Reagents.—All chemicals were prepared in concentrated stock solution which had been neutralized to pH 7 with dilute potassium hydroxide. Prior to use these were diluted to the required concentration with isotonic sucrose phosphate solution (9 parts of 0.25 M sucrose and 1 part of 0.1 M potassium phosphate buffer, pH 7.1.

[1] M. R. Bovarnick, personal communication.

The component solutions were sterilized by autoclaving and cooled before admixing). The same medium was used for the washing and final suspension of rabbit erythrocytes.

The diluent for human erythrocytes was sucrose–PO_4–Mg, prepared by adding 10 ml. of autoclaved 0.1 M $MgCl_2$ solution to 100 ml. of sucrose–PO_4 solution.

Adenosinetriphosphate (ATP)[2].—Pabst ATP–disodium salt was used. Both the crystalline and non-crystalline products proved equally effective in our system.

Hemolysate of Rabbit Erythrocytes.—Rabbit blood obtained by heart puncture was mixed with an equal volume of sterile citrate-dextrose solution (sodium citrate, 0.073 M; citric acid, 0.0024 M; and glucose, 0.113 M). After centrifugation and removal of the plasma and the buffy coat, the cells were washed three times with 4 volumes of sucrose–PO_4 and resuspended to a concentration of 8 per cent in the same diluent. These cells, stored at 0 to 4°C., produced active hemolysate for 3 weeks—the longest period tested.

Hemolysate was prepared as follows: 4 ml. of the above 8 per cent rabbit cells were centrifuged, the supernatant fluid discarded, and 4.8 ml. of water was added to lyse the packed erythrocytes. After 5 minutes 1.2 ml. of 42.5 per cent sucrose was added to render the solution isotonic and the hemolysate was centrifuged at 25,000 *G* at 4°C. for 10 minutes to bring down the greater part of the stroma. The supernatant fluid, which contained all the activity of the original hemolysate, was used in the hemolysin test. The hemolysate was prepared on the day of use although it retained some of its activity for as long as a week when kept at 4°C., and was fully active for 2 weeks when quickly frozen and stored at −70°.

Human Erythrocytes.—Human blood in ACD solution was obtained from a Blood Bank 1 or 2 days after drawing. Although all blood groups proved suitable, as routine group O blood was used. The blood was centrifuged and the plasma and buffy coat were discarded. The packed cells were then washed three times with 4 volumes of sucrose–PO_4–Mg solution and finally suspended to a concentration of 8 per cent in the same diluent. The cells were stored at 0 to 4° and discarded after 2 weeks.

Hemolysin Test.—The procedure followed was essentially the one described by Synder *et al.* (1) except that the incubation period was reduced to 1 hour since maximal hemolysis was achieved in this time.

Into Kahn tubes kept in an ice bath were placed 0.05 ml. of 0.002 M versene, 0.05 ml. of 0.008 M ATP, and aliquots of rabbit cell hemolysate ranging from 0.02 to 0.14 ml. Sucrose–PO_4 was added to bring the total volume to 0.3 ml. Then 0.3 ml. of an 8 per cent suspension of human erythrocytes in sucrose–PO_4–Mg solution was added to each tube. Proper control tubes, lacking ATP and rabbit hemolysate respectively, were set up at the same time, the latter serving as an instrument blank against which all other tubes were read. The tubes were then placed in a 34° C. water bath for 1 hour. After incubation, 2 ml. of isotonic saline was added to each tube, the

[2] The following abbreviations are used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosinemonophosphate; CTP, cytidine triphosphate; GSH, glutathione; GSSG, oxidized glutathione; DPN, diphosphopyridine nucleotide, TPN, triphosphopyridine nucleotide; FMN, flavine mononucleotide; FAD, flavine adenine dinucleotide; versene, ethylene-diaminetetraacetic acid; Tris, tris (hydroxymethyl) amino methane.

tube contents were well mixed, and then centrifuged at 2,500 R.P.M., for 10 minutes. The optical density at 545 mμ was measured in a Coleman Jr. spectrophotometer against the instrument blank described above.

The O.D. readings were corrected for the hemoglobin value contributed by various aliquots of the rabbit hemolysate by subtracting the optical density obtained in the control tube (lacking ATP) from that obtained in the corresponding complete system (with ATP). The per cent hemolysis was calculated by dividing the corrected O.D. readings by the optical density obtained by complete lysis of 0.3 ml. of 8 per cent human erythrocyte suspension with 2.3 ml. of water. Per cent hemolysis *vs.* rabbit hemolysate concentration is plotted in Fig. 1. In studies on the effect of various

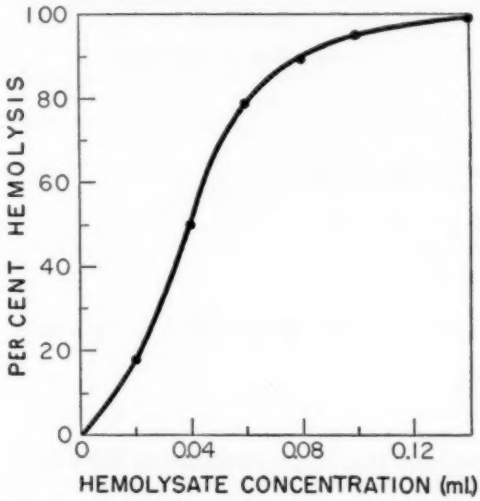

FIG. 1. Hemolysis of human erythrocytes by rabbit red cell hemolysate. Each tube contained 0.3 ml. of 8 per cent human erythrocytes in sucrose–PO_4–Mg; ATP (6.67×10^{-4} M); versene (1.67×10^{-4} M); and rabbit hemolysate as indicated. Diluent is sucrose–PO_4. Total volume = 0.6 ml.

additional agents on the system, that amount of rabbit hemolysate which resulted in the hemolysis of 50 per cent of the human erythrocytes without added agents was used.

Experimental and Results

Centrifugation of Rabbit Hemolysate.—When a hemolysate of rabbit red cells made isotonic with sucrose was subjected to centrifugation at 25,000 *G* for 30 minutes at 4° in the high speed attachment of the International refrigerated centrifuge, the resultant precipitate was inactive and the total hemolytic activity of the original hemolysate remained in the supernatant fluid. However, centrifugation even at low speed (146 *G*) of rabbit hemolysate rendered isotonic with saline resulted in the precipitation of 60 per cent of

the lytic factor as shown in Table I. The remainder of the activity was retained in the supernatant fluid and could not be brought down by further centrifugation at speeds of up to 25,000 *G* for 30 minutes.

If the active precipitate, P-1, was washed by suspension in saline—0.01 M PO_4 and centrifuged at 1929 *G* for 10 minutes only 25 per cent of the original activity of P-1 was precipitated. However, if P-1 was suspended in sucrose—PO_4 and centrifuged as above the resultant precipitate, P-2, contained 60 per cent of the original activity of P-1 (Table I). The final method evolved for obtaining the active precipitated fraction was as follows: 0.032 ml. of packed

TABLE I

Centrifugation of Lytic Factor

Fraction	Hemolytic activity
	per cent
1. Original hemolysate in isotonic saline	100
2. Centrifuged at 146 *G* for 10 min.	
(*a*) Supernatant fluid (S-1)	33
(*b*) Precipitate (P-1)	63
3. P-1 suspended in saline–0.01 M PO_4 and centrifuged at 1929 *G* for 10 min.	
(*a*) Supernatant fluid (S-2)	47
(*b*) Precipitate (P-2)	16
4. P-1 suspended in sucrose–0.01 M PO_4 and centrifuged at 1929 *G* for 10 min.	
(*a*) Supernatant fluid (S-2)	25
(*b*) Precipitate (P-2)	38

rabbit erythrocytes was lysed with 4.8 ml. water and 1.2 ml. of 4.5 per cent NaCl was then added to restore isotonicity. The hemolysate was centrifuged at 1929 *G* at 4°C. for 10 minutes, the supernatant fluid discarded, and the precipitate, P-1, was washed by suspension in 6 ml. of sucrose—PO_4 and recentrifugation at the same speed, 1929 *G*. The supernatant fluid was discarded and the second precipitate, P-2, was suspended in 3 ml. of sucrose—PO_4 (half the original volume) for use in the hemolysin studies. This preparation, hereafter referred to as rabbit P-2, was relatively free of hemoglobin and of the other soluble factors (glutathione, ergothionine, etc.) present in the original hemolysate, a decided advantage when studying the effect of these components on the hemolytic system. It could be preserved at −70°C. and was fully active after a storage period of 1 month. Further purification of the hemolytic factor is currently underway.

Absolute Requirements.—Rabbit red cell hemolysate did not lyse human erythrocytes unless both Mg^{++} and ATP were present. Mg^{++} at final concentration of 5×10^{-4} to 5×10^{-3} M gave maximal hemolysis in the presence of 1.67×10^{-4} M ATP. The response to increasing concentrations of ATP is shown in Fig. 2.

It can be seen that maximal hemolysis is obtained with 1.67×10^{-4} M ATP. A fourfold excess, namely 6.67×10^{-4} M, was used in all subsequent experiments to insure fulfillment of the ATP requirement. If more ATP was added

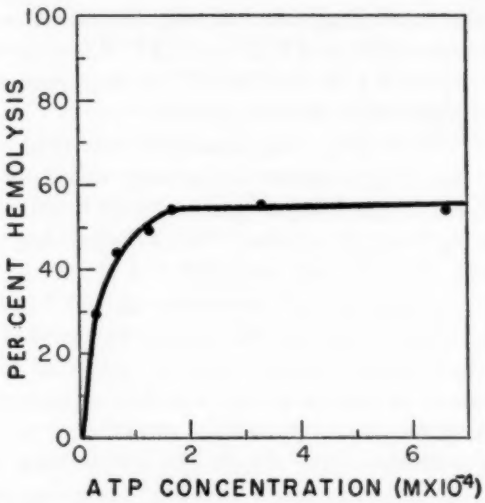

FIG. 2. Effect of ATP concentration on hemolysis. Each tube contained 0.04 ml. of rabbit hemolysate; 0.3 ml. of 8 per cent human erythrocytes in sucrose–PO–Mg; versene (1.67×10^{-4}M); and the given amount of ATP. Diluent is sucrose–PO_4. Total volume = 0.6 ml.

to a system which had been incubated for 1 hour with 6.67×10^{-4} M ATP, and the enriched system was then incubated for an additional hour, no further hemolysis occurred.

Adenine, adenosine, or inorganic pyrophosphate at a concentration of 3.3×10^{-4} M neither caused hemolysis in the absence of ATP nor inhibited the system in the presence of an equal concentration of ATP. Adenylic acid (AMP) was found to inhibit hemolysis in the presence of 6.67×10^{-4} M ATP, the inhibition being proportional to the AMP concentration, namely, 0 per cent inhibition at 3.34×10^{-4} M AMP, 20 per cent at 6.67×10^{-4} M AMP, and 50 per cent at 1.33×10^{-3} M AMP.

Adenosinediphosphate (ADP) was quite effective in promoting hemolysis. Maximal activity, which was reached at 6.67×10^{-4} M ADP, averaged 60

per cent of the control maximal hemolysis value obtained with ATP (6.67×10^{-4} M), and the same rabbit hemolysate preparation. When both ADP and ATP were added simultaneously to the system, the resultant hemolysis was equal to that obtained with ATP alone.

Cytidine triphosphate (CTP), and uridine triphosphate (UTP) caused slight hemolysis (about 25 per cent of the maximal value obtained with ATP) when tested at a concentration of 4×10^{-4} M. CTP and UTP added together were about 50 per cent as effective as ATP. The presence of 1.33×10^{-5} M ATP, which in itself produced no hemolysis, almost doubled the activity of UTP and CTP when used singly but had very little effect on hemolysis produced by simultaneous addition of UTP and CTP. When the system contained maximal concentrations of ATP (6.67×10^{-4} M) hemolysis was not enhanced by the addition of either UTP or CTP or both.

Effect of Dialysis and Heating.—The hemolytic activity of rabbit hemolysate was not affected by dialysis against an isotonic saline solution containing 0.01 M PO_4 buffer, pH 7, and 0.001 M versene, for 18 hours, at 4°C. Moreover, no lysis of human erythrocytes occurred when a dialysis bag containing rabbit hemolysate, versene (2×10^{-3} M), and ATP (3.4×10^{-4} M) was placed in a test tube containing human red cells in versene and ATP (also at 2×10^{-3} M and 3.4×10^{-4} M, respectively) and the system was incubated for 3½ hours at 34°C. These observations, coupled with the fact that the hemolysate is completely inactivated by heating at 56°C. for 1 hour, suggest that the active principle is a large molecule and probably a protein.

Inhibitions and Activators.—(1) Metals. All metals were tested in a final concentration of 5×10^{-4} M, at which level Mg^{++} produced maximal hemolysis. In the absence of Mg^{++}, both Mn^{++} and Co^{++} showed some activity, amounting to 21 and 41 per cent respectively, of the hemolysis produced by Mg^{++}, Ca^{++}, Fe^{++}, and Fe^{+++}, could not substitute for Mg as activators. The following metals proved inhibitory in the presence of a tenfold excess of Mg^{++} (5×10^{-3} M); Ca^{++} (21 per cent inhibition), Mn^{++} (30 per cent), Fe^{++}, (16 per cent), Zn^{++} (100 per cent), Hg^{++} (100 per cent). Cu^{++} caused hemagglutination of the human erythrocytes.

In this connection it is interesting to note that human urine inhibited hemolysis, apparently by virtue of its metallic cations, since the inhibitory factor was removed by exhaustive dialysis of the urine and was overcome by versene. Moreover, the inhibitor was not destroyed by sulfuric acid digestion of the urine.

The hemolysin test is carried out in the presence of 1.67×10^{-4} M versene in order to chelate any trace of metal contaminants. A higher versene concentration was not employed since it in itself tended to lyse human red cells.

(2) Organic compounds. Eserine (physostigmine) in a concentration of 1×10^{-4} M neither inhibited nor activated the system.

Cysteine (1.67×10^{-4} M) caused a 64 per cent inhibition of hemolysis by rabbit hemolysate but glutathione (GSH) in high concentrations, (1.67×10^{-3} to 1.67×10^{-2} M), increased the hemolytic activity by about 20 per cent. Oxidized glutathione (GSSG) at 1.67×10^{-3} M caused 84 per cent inhibition and this was almost completely overcome by the simultaneous addition of a tenfold excess of GSH.

The hemolytic activity of freshly prepared, highly active, rabbit P-2 was enhanced only to a slight extent by GSH when versene (1.67×10^{-4} M) was present. In the absence of versene, however, 1.7×10^{-2} M GSH increased hemolysis by about 30 per cent and this was equal to the activation obtained

TABLE II

Activation of Rabbit P-2 by Glutathione

GSH concentration	Hemolysis	Activation
moles	*per cent*	*per cent*
0	6	—
1.7×10^{-6}	6	0
1.7×10^{-5}	7	0
1.7×10^{-4}	6	0
1.7×10^{-3}	21	350
1.7×10^{-2}	41	683

Each tube contained 0.1 ml. of rabbit P-2 which had been aged for 24 hours at 4° before use; versene (1.67×10^{-4}M); ATP (6.67×10^{-4}M); 0.3 ml. of 8 per cent human erythrocytes in sucrose–PO_4–Mg; and sucrose–PO_4 or GSH as indicated. Total volume = 0.6 ml.

by the addition of versene alone. In this case glutathione may have merely served to combine with trace metal inhibitors in the system, as did versene, and this resulted in an increased hemolytic effect.

However, weakly active rabbit P-2 preparations, obtained by storage for several days at 4° or by preincubation for 1 hour at 34°, showed a pronounced response to GSH which was independent of the presence of versene as shown in Table II.

In Fig. 3 the inhibition of freshly prepared rabbit P-2 by GSSG in a concentration of from 3.3×10^{-4} M to 1.67×10^{-3} M is graphically presented. As in the case of rabbit hemolysate, inhibition of rabbit P-2 by GSSG (1.67×10^{-3} M) was almost completely overcome by ten times the concentration of GSH (1.67×10^{-2} M).

L-Ergothionine (1.7×10^{-3} M to 1.7×10^{-2} M) also completely inhibited the system, showing no effect at lower concentrations. Here again GSH was effective in partially negating the inhibition. In the presence of 1.67×10^{-2} M GSH an equal concentration of L-ergothionine reduced hemolysis by only 50 per cent and 1.67×10^{-3} M ergothionine caused only 23 per cent inhibition.

DPN, TPN, FMN (all at 1×10^{-4} M), FAD (2×10^{-5} M), and coenzyme-A (9×10^{-5} M) were without effect on the hemolytic end-point. When coenzyme-A was added in combination with 1.7×10^{-3} M GSH no activation above that of the GSH alone was observed.

Metabolic Inhibitors.—When various metabolic inhibitors at a final concentration of 0.002 M were added to our system it was found that hemolysis was reduced by sodium cyanide (54 per cent inhibition), α-dinitrophenol (22 to 40 per cent), and sodium azide (10 per cent). Potassium fluoride inhibited the

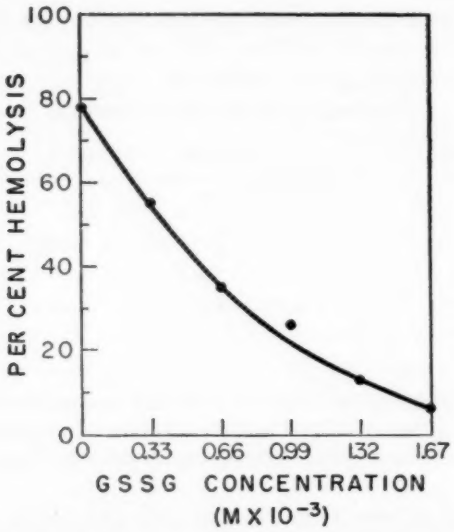

FIG. 3. Inhibition of hemolysis by oxidized glutathione. Each tube contained 0.15 ml. of freshly prepared rabbit P-2; versene (1.67×10^{-4}M); ATP (6.67×10^{-4}M); 0.3 ml. of 8 per cent human erythrocytes in sucrose–PO_4–Mg; and GSSG as indicated. Diluent is sucrose–PO_4. Total volume = 0.6 ml.

system completely at 0.04 M but had no effect at 0.004 M. Iodoacetate tested at 0.005 M and 0.001 M resulted in 85 and 53 per cent inhibition, respectively.

Inhibition by Human Stroma.—Since it appeared plausible that in our system the lysis of human erythrocytes by rabbit hemolysate was mediated by some reaction involving the membrane of the human red cell, the effect of human stroma on the hemolytic end-point was investigated.

Stroma was prepared as follows: 1.2 ml. of packed, saline-washed, human erythrocytes were lysed by the addition of 12 ml. of water and the stroma was separated by centrifugation at 5000 R.P.M. for 15 minutes at 4°. The supernatant fluid was discarded and the stroma was washed twice with 4 volumes of saline and was then suspended in saline to a final volume of 5 ml.

Thus, 0.1 ml. of this preparation contained the stroma derived from 0.024 ml. of packed cells which was equivalent to the concentration of human erythrocytes used in the hemolysin test (0.3 ml. of 8 per cent cells). In all cases, the stroma was prepared from the same batch of cells as were subsequently used in the hemolysin test.

In these experiments the effect of 0.1 ml. of freshly prepared human stroma on rabbit hemolysate which had been titrated to a hemolytic end-point of approximately 50 per cent was tested with five different human stroma preparations as follows:—

1. Rabbit hemolysate and human erythrocytes, in the presence of 6.67×10^{-4} M ATP, were incubated according to the usual procedure with and without added stroma.

2. Rabbit hemolysate alone, and hemolysate plus human stroma, were preincubated for 1 hour at 34°. At the end of this time, ATP (6.67×10^{-4} M) and human erythrocytes in sucrose—PO_4—Mg were added and the complete systems were then incubated for an additional hour.

3. Rabbit hemolysate alone, and hemolysate plus human stroma, were preincubated for 1 hour in the presence of 6.67×10^{-4} M ATP. After preincubation, human erythrocytes in Su—PO_4—Mg, and ATP to readjust the final concentration to 6.67×10^{-4} M were added and incubation was continued as in (2).

In Table III the data obtained from five separate experiments using different preparations of stroma, red cells, and rabbit hemolysate are recorded.

Hemolysis is almost always inhibited (by about 20 per cent) by human stroma prepared from the same batch of cells as are used in the hemolysin test itself. Preincubation of rabbit hemolysate and stroma prior to the addition of human erythrocytes and ATP to the system, enhances the effect. Preincubation in the presence of ATP results in almost complete inhibition of hemolysis. Although we have found that rabbit hemolysate contains ATPase, the reduced lysis cannot be attributed to a low ATP concentration resulting from destruction during preincubation since, after preincubation, an amount of ATP equal to at least twice the concentration necessary for full hemolysis is supplied to the system at the same time that erythrocytes are added.

Adenosinetriphosphate Balance Studies.—In an attempt to elucidate the role of ATP in the hemolytic process the concentration of labile phosphate was followed during the course of hemolysis.

Freshly prepared rabbit P-2 was incubated with human red cells in the presence of versene, Mg^{++}, and ATP, and aliquots of the incubation mixture were assayed for the extent of hemolysis and for labile phosphate immediately before and after 30 and 60 minutes' incubation at 34°. At the same time, controls containing (1) human erythrocytes, (2) rabbit P-2, and (3) human he-

molysate were set up. In this experiment sucrose-0.03 M Tris, pH 7.2, was substituted for the usual sucrose—PO_4, both in the washing and preparation of human cells and of rabbit P-2, and as the diluent for the hemolysin test.

At the desired time the reaction was arrested by rapid chilling in an ice bath and the mixture was centrifuged for 10 minutes at 4°. The supernatant fluid was carefully separated from the cellular residue, the cells were resus-

TABLE III

Inhibitory Effect of Human Stroma on Hemolysis

Experiment No.	Per cent hemolysis					
	No Preincubation		Preincubation — ATP		Preincubation + ATP	
	Hemolysate alone	Stroma + hemolysate	Hemolysate alone	Stroma + hemolysate	Hemolysate alone	Stroma + hemolysate
1*	66	54	65	37	65	3
2*	52	37	54	36	56	13
3‡	55	44	37	31	32	4
4§	57	40	57	21	52	9
5§	63	54	69	30	69	2
Average per cent inhibition..		19		46		93

* Experiments 1 and 2. The final ATP concentration in the hemolysin test and in preincubation with ATP is 6.67×10^{-4} M. Mg^{++} is not present during the preincubation period but is added with the red cells (0.3 ml. of 8 per cent cells in $SuPO_4$–0.01 M $MgCl_2$) after preincubation.

‡ Experiment 3. The conditions are the same as above but, in addition, versene (1.67×10^{-4} M final concentration) is present during both the preincubation period and the hemolysin test.

§ Experiment 4 and 5. The final ATP concentration in the hemolysin test and in preincubation with ATP is 1.33×10^{-3} M. Mg^{++} (5×10^{-3} M final concentration) and versene (1.67×10^{-4} M final concentration) are present during the preincubation period and in the hemolysin test.

pended to the original volume with ice cold saline, and both fractions were deproteinized by the addition of ice cold trichloracetic acid (10 per cent final concentration). In the rabbit P-2 control, the centrifugation step prior to deproteinization was omitted since the resulting precipitate was very small and difficult to separate and the mixture was analyzed as a whole. The concentrations of labile phosphate in the protein-free filtrates were calculated as the difference between the inorganic phosphate values before and after hydrolysis in 1 N HCl for 7 minutes, using the procedure of Fiske and SubbarRow (2). The results of a typical experiment are shown in Table IV.

Since human erythrocytes contain labile phosphate compounds, the flasks to which human cells or hemolysate had been added were found to contain

about 16 per cent more than the theoretical amount of labile phosphate which would arise from the added ATP alone. After 30 minutes of incubation the complete system (flask 4) showed that 19 per cent of the erythrocytes had been hemolyzed and that the total labile phosphate content of the combined supernate and the precipitate had decreased by a total of 18 per cent of the original value. The human hemolysate control decreased by only 3 per cent

TABLE IV

Change in Labile Phosphate during Hemolysis

Flask	Contents	Total labile phosphate								
		0 min.			30 min.			60 min.		
		Super.	Ppt.	Total	Super.	Ppt.	Total change	Super.	Ppt.	Total change
		μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols
1	Human erythrocyte control	15.44	3.46	18.90	16.09	3.46	+0.65	15.23	3.02	−0.65
2	Rabbit P-2 control	15.01	—	15.01	11.50	—	−3.51	11.07	—	−3.94
3	Human hemolysate control	17.66	1.30	18.96	17.82	0.65	−0.49	17.28	0.65	−1.03
4	Human erythrocytes + rabbit P-2	14.42	3.19	17.61	12.74	2.11	−2.76	12.20	1.13	−4.28
		0 per cent hemolysis			19 per cent hemolysis			61 per cent hemolysis		

All flasks contain a final concentration of 1.67×10^{-4} M versene, 5×10^{-3} M $MgCl_2$, and 2.96×10^{-4} M ATP. In addition, the following are added to the respective flask:

1. 7.25 ml. of 16 per cent human erythrocytes.
2. 11.25 ml. of rabbit P-2.
3. 7.25 ml. of hemolyzed 16 per cent human erythrocytes restored to isotonicity with sucrose.
4. 7.25 ml. of 16 per cent human erythrocytes and 11.25 ml. of rabbit P-2.

The diluent throughout is isotonic sucrose–0.03 M-Tris, pH 7.2.

Total volume = 27 ml. of which 5 ml. aliquots are analyzed at the stated time.

while the erythrocyte control actually increased by 3 per cent. However, in the case of rabbit P-2 alone, 23 per cent of the labile phosphate had disappeared.

Similarly, after 60 minutes when the complete system showed 61 per cent hemolysis, the total decrease in labile phosphate (24 per cent) was about the same as that in the rabbit P-2 control (26 per cent), the human erythrocyte and human hemolysate control values being decreased by 3 and 5 per cent, respectively, of the original value. It appeared, therefore, that the decrease in labile phosphate during hemolysis of human erythrocytes by rabbit P-2 was due to the activity of the rabbit preparation alone and that there was essentially no net change in total labile phosphorus in the hemolyzing process.

These results were duplicated in another experiment in which the concentration of ATP alone, instead of total labile phosphate, was followed. The incubation procedure was the same except that sucrose—PO_4 was the diluent throughout and deproteinization was effected by ice cold perchloric acid (2 per cent final concentration). The resulting protein-free filtrates were neutralized with potassium hydroxide to pH 7.4 (phenol red used as internal

TABLE V

Change in ATP Concentration during Hemolysis

Flask	Contents	Total ATP								
		0 min.			30 min.			60 min.		
		Super.	Ppt.	Total	Super.	Ppt.	Total change	Super.	Ppt.	Total change
		μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols
1	Human erythrocytes control	6.62	0.86	7.48	6.34	0.61	−0.53	5.94	0.58	−0.96
2	Rabbit P-2 control	5.29	—	5.29	4.21	—	−1.08	3.02	—	−2.27
3	Human hemolysate control	6.19	0.25	6.44	6.01	0.18	−0.25	6.19	0.18	−0.07
4	Human erythrocytes + rabbit P-2	5.94	0.65	6.59	4.97	0.58	−1.04	3.99	0.36	−2.24
		0 per cent hemolysis			22 per cent hemolysis			51 per cent hemolysis		

All flasks contain a final concentration of 1.67×10^{-4} M versene, 5×10^{-3} M $MgCl_2$, and 3.33×10^{-4} M ATP. In addition, the following are added to the respective flask:

1. 4.50 ml. of 16 per cent human erythrocytes.
2. 8.00 ml. of rabbit P-2.
3. 4.50 ml. of hemolyzed 16 per cent human erythrocytes restored to isotonicity with sucrose.
4. 4.50 ml. of 16 per cent human erythrocytes and 8.00 of rabbit P-2.

The diluent throughout is sucrose–PO_4.

Total volume = 18 ml. of which 5 ml. aliquots are analyzed at the stated time.

indicator) and the insoluble potassium perchlorate was removed by centrifugation at 4°. Aliquots of the perchlorate-free supernatant fluids were then analyzed enzymatically for ATP by the method of Kornberg (3) in which glucose, TPN, glucose—6—PO_4 dehydrogenase, and hexokinase are added to the system and the reduction of TPN is followed spectrophotometrically at 340 mμ using a Beckman model DU quartz spectrophotometer. Glucose—6—PO_4 dehydrogenase[3] was prepared according to Kornberg (4) and hexokinase[3] was made following the method of Berger *et al.* (5) to step 3. The data are given in Table V.

[3] We wish to thank Dr. Marianna R. Bovarnick for her kindness in providing these enzymes.

It can be seen that the loss of ATP after 30 and 60 minutes of incubation of combined rabbit P-2 and human erythrocytes when 22 and 51 per cent hemolysis, respectively, had been achieved, was equal to the decrease obtained in the rabbit P-2 control. These results show that the action of the rabbit factor on human red cells, which leads to hemolysis of the latter, is associated with no net decrease of ATP in the system but that the observed loss is equal to the endogenous ATP-ase activity of the rabbit preparation. Further studies to gain insight into the chemical reactions whereby ATP is utilized in the hemolytic process are currently under way employing isotopically labelled ATP.

DISCUSSION

The requirement for ATP, normally an intracellular source of energy for synthetic processes, in a hemolytic reaction, generally considered a degradative process, seems somewhat paradoxical. The precise nature of the reactions involved remains to be determined. One interesting possibility is that ATP is involved as an intermediary in a reaction between rabbit hemolysate and a receptor site on the red cell membrane. The presence of reactive sites for ATP has been postulated on red cell and other cell surfaces as part of the mechanisms involved in the transfer of both cations (6, 7) and phosphate (8) across the cell membrane. In both instances it has been hypothesized that the reactive transfer site on the cell surface utilized high energy phosphate which had been generated inside the cell as the driving force for the directional transfer of the ions under consideration.

In the case described in this paper an additional potential source of energy in the form of extracellular ATP is brought to bear on the system. If ATP does function here as a source of energy, the possibility arises that the reactive site in this system might be the same as that involved in normal ion transfer processes, but it is conceivable that the new condition might upset the normal balance and direction of ion transport in such fashion as to produce osmotic disturbances leading to hemolysis. Similar considerations might apply to the functioning of a "water pump" if this actually exists. Another possibility is that phosphorylation of the membrane destroys its integrity. The possibility that the acetylcholine–choline esterase system mechanism, known to be present in human red cells and to influence ion transport (9–11), is involved in our system seems to be ruled out by the failure of physostigmine, a choline esterase inhibitor, to affect our system.

The lytic factor present in rabbit hemolysate appears to be an enzyme since it is stable to dialysis and is destroyed at 56°C. Moreover, the marked activation of the system by glutathione, in conjunction with its sensitivity to oxidized glutathione, iodoacetate, and heavy metals, suggests the involvement of a sulfhydryl enzyme.

These various aspects, as well as the further interesting one of the relation of this phenomenon to "ATP shock" (12) are under investigation.

SUMMARY

1. Hemolyzed rabbit cells contain a factor which lyses human erythrocytes *in vitro* when Mg^{++} and certain nucleotides are supplied to the system. Of the nucleotides tested ATP is the most active, although no net loss of ATP or of labile phosphate seems to be associated with the hemolytic process.

2. The lytic factor appears to be a sulfhydryl enzyme which attacks the membrane of the human red cell, its hemolytic activity being inhibited by human stroma.

3. The system is activated by glutathione and is inhibited by heavy metals, oxidized glutathione, cysteine, ergothionine, and a number of metabolic inhibitors. Physostigmine has no effect.

4. Partial purification of the lytic factor has been achieved by fractional centrifugation.

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CHANGES IN MEMBRANE CHARACTERISTICS OF HEART MUSCLE DURING INHIBITION*

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(Received for publication, February 27, 1956)

INTRODUCTION

A change in the membrane characteristics of heart muscle during vagal inhibition has been suggested by numerous experiments. In 1887 Gaskell (8) had already reported an increase in demarcation potential of the turtle auricle on stimulation of the vagus. A second phenomenon found by Samojloff (20) and frequently confirmed by other authors was a shortening of the auricular action potential during inhibition (*cf.* references 2, 19, 22, and 25, for extensive references). More recently, using intracellular recording methods Burgen and Terroux (3) and Hoffman and Suckling (10) confirmed that the recovery phase of the heart muscle action potential was greatly accelerated by vagal stimulation or by external application of acetylcholine. Since then inhibitory processes during vagal activity have been studied in detail in simultaneous investigations by del Castillo and Katz (4) and by Hutter and Trautwein (14). Their results, as well as those of Burgen and Terroux (3) on the effect of acetylcholine on the resting potential, indicate that the inhibitory transmitter, *i.e.* acetylcholine, changes the permeability to specific ions, thereby leading to the observed membrane changes. In these studies no definite conclusions regarding conductance or resistance changes could be reached.

The first more direct evidence for a decrease of the membrane resistance during inhibition was shown in the crustacean neuromuscular junction. Fatt and Katz (7) found that the restitution phase of the excitatory end plate potential was accelerated by inhibitory impulses, indicating a shortened time constant of the membrane. In studies on spinal motoneurons of the cat, Coombs, Eccles, and Fatt (5) came to the conclusion that permeability changes to K^+ and Cl^- ions may be responsible for the membrane phenomena of inhibition. In the isolated nerve cells of crayfish and lobster stretch receptors, additional evidence was obtained that neural inhibition increases the conductance of the cells (17); here also Cl^- and K^+ have now been shown to participate in the inhibitory potential (Hagiwara and Edwards, unpublished observations).

* Supported by a grant from the United States Public Health Service.

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In view of the available information from various cells in different species, pointing to a common synaptic mechanism, it seemed desirable to attempt a more detailed approach in measuring membrane characteristics during inhibition. Therefore changes in the length constant and time constant of fiber bundles in the frog's auricle were measured during the application of acetylcholine. Absolute values of membrane resistances were difficult to obtain since the heart muscle fibers form a syncytium and therefore cannot be treated as uniform cylindrical structures, as are nerve axons or skeletal muscle fibers to which the present methods are directly applicable (9, 16). The results give, however, direct evidence that the membrane resistance during inhibitory activity is greatly reduced.

Method

Frog auricles were opened and fine cylindrical preformed strips were carefully excised under a dissecting microscope. The strips were about 5 mm. long and 0.1 mm. in diameter and had to be "clean," to avoid adhering of fluid droplets at least in the region in which recording was done. Special care was taken to avoid injury to the thin muscle bundles which were held in fine forceps at each end. The small bundles of auricle muscle did not usually beat spontaneously. The state of the preparation was judged by the size of action potentials and the vigorous accompanying contractions in response to stimulation. The amplitude of contractions usually did not decline for 5 to 10 hours. For recording the strips were raised into paraffin oil above the Ringer bath. Separate containers holding various test solutions were placed below the paraffin oil layer and the preparations were lowered into them for various periods, with the electrodes remaining in place. On occasions a drop of acetylcholine in Ringer was applied directly to a portion of the strip by a pipette, and after 20 to 50 seconds the drop was removed, the preparation remaining in paraffin oil. This method of application caused the least disturbance in recording conditions, and minimized variations in the quantity of fluid adhering to the strip due to solution changes. All measurements were made in non-beating preparations.

Four silver-silver chloride electrodes were connected to the preparation through agar-Ringer and through bristles from a "natural" bristle tooth-brush which were first boiled and then cut down to a fine tip which made contact with the muscle bundle over an area about 0.1 mm. wide.

Hodgkin and Rushton's (9) technique of measuring time constants by applied square pulses was used with the circuit modification described by Katz (16). Square pulses of current were passed through two electrodes, one near the end and one near the middle of the muscle bundle. The amount of current passed through the preparation was monitored on one beam of the oscilloscope. One of the recording leads was fixed near the end of the strip while the other was movable. The pulses were recorded with a conventional D.C. amplifier. The distance between the movable recording electrode and the nearby stimulating electrode was measured with a calibrated microscope eyepiece.

Validity of Assumptions.—The application of the cable equation to a strip of heart muscle must be made with caution. The analysis of Hodgkin and Rushton (9) for single fibers was used by Katz (16) for the study of aggregates of muscle fibers.

He confirmed his results on individual muscle as well as on nerve cells. The measurements reported here give an average value for the membrane constants of heart tissue and variations in the values of these constants caused by acetylcholine give a good indication of the direction of the changes. However, conversion of the results into quantitative values of the resistance changes of the muscle membrane rests on numerous assumptions.

In the ideal case, as treated by Hodgkin and Rushton, it is assumed that the current flow is parallel in the internal and external conductors which obey Ohm's law. Further, the electrode width should be negligible and the interelectrode distance infinite. In our experiments the characteristic length in Ringer was 3 to 5 times the diameter of the bundle, so that the current flow must have been nearly parallel (16). In the presence of acetylcholine the space constant was as little as twice the diameter and here the analysis becomes more questionable. The assumption of ohmic resistances was confirmed by measurements of the interelectrode potential gradient; the current was kept below the half threshold value to minimize any rectification that may have occurred due to "local" responses. The electrode width was one-fifth or less of the space constant in Ringer but only about one-third in acetylcholine; this must have introduced a slight "smearing" of the potentials. The distance between the two stimulating electrodes and the distance between the two recording electrodes were 10 or more characteristic lengths, so that interference between them could not have been important.

RESULTS

Measurement of the Characteristic Length of the Membrane (λ).—The maximal heights of the electrotonic potentials which were set up by the subthreshold square pulses were measured at different distances between the stimulating cathode and the roving recording lead. The steady state height of the electrotonic pulse (anodal as well as cathodal) is an exponential function of the interelectrode distance; thus a plot of the pulse height as a function of distance on a semilogarithmic scale gives the characteristic length directly. Fig. 1 shows some of the electrotonic potentials at different distances from the stimulating cathode. Between *A* and *B* a drop of acetylcholine (5.5 μM/liter) together with prostigmin (30 μM/liter) was applied for 30 seconds and then removed. The potentials in *B*, taken within 5 to 6 minutes of the drug application, clearly became smaller and at a distance of 0.6 mm. they could not be measured even with an increased amplification (note calibration differences). After washing in ordinary Ringer solution for about 5 minutes the original potential size was restored (Fig. 1 *C*). The complete results of the experiment of Fig. 1 are plotted in Fig. 2. The reversible decrease in characteristic length was found in all applications (Table I). In one case (strip two, Table I), for unknown reasons, the change was small, but a second application produced the usual effect. The change in λ was confirmed in numerous repeated acetylcholine applications without prostigmin; however, the changes were short lasting (see below). In the present experiments, the threshold concentration

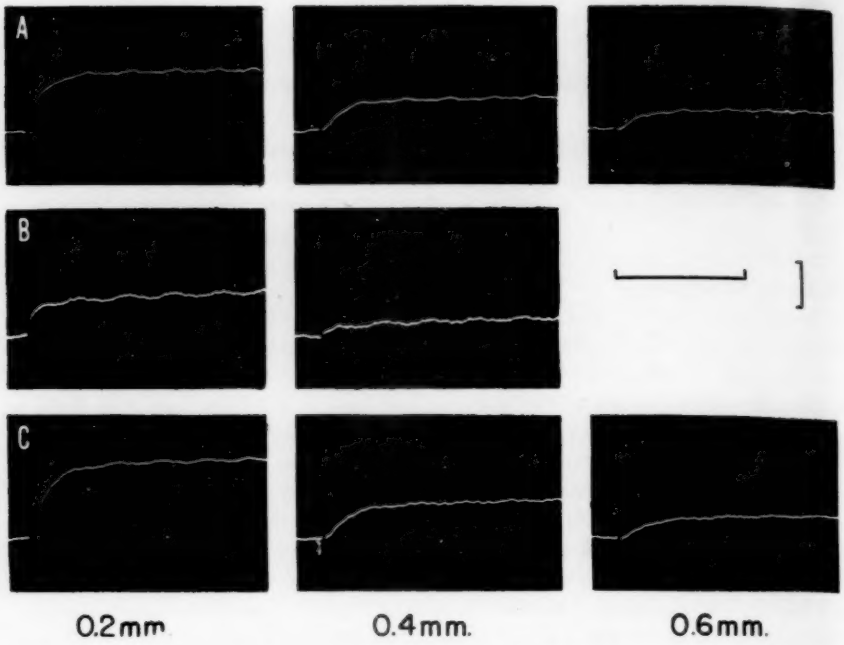

FIG. 1. Electrotonic pulses at different interelectrode distances. *A*, normal records. *B*, during action of 5.5 μM/liter acetylcholine and 30 μM/liter prostigmin. *C*, after washing the preparation in Ringer for 5 minutes. Time calibration 50 msec. Amplification, 1 mv. for lines *A* and *C*, ½ mv. for *B*.

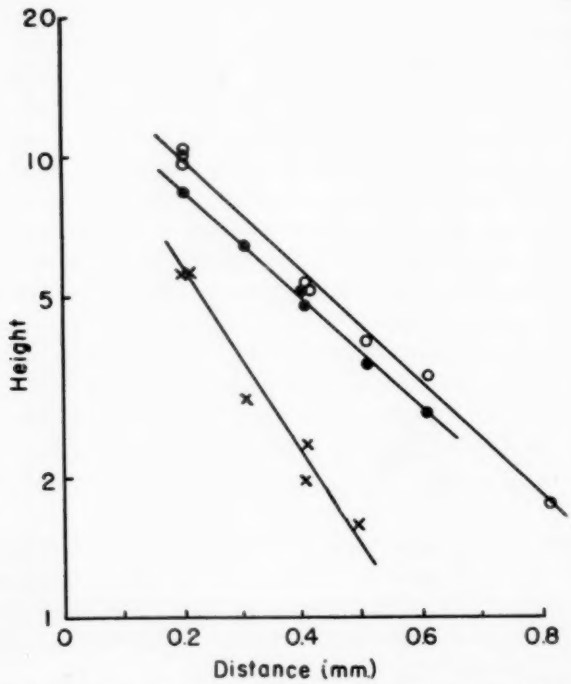

FIG. 2. Semilogarithmic plot of pulse height as a function of interelectrode distance. Closed circles, preparation normal. Crosses, during action of 5.5 μM/liter acetylcholine and 30 μM/liter prostigmin. Open circles, after washing in normal Ringer. Same experiment as Fig. 1.

of acetylcholine (tested by time and length constant changes and by shortening of conducted muscle potentials) was about 0.55 μM/liter (1 part in 10^7) without prostigmin. Almost all tests were done with concentrations of 5.5 μM/liter acetylcholine (1 part in 10^6) and 30 μM/liter prostigmin.

TABLE I

Effect of Acetylcholine on Membrane Constants

	λ	τ_{m1}	τ_{m2}
	mm.	msec.	msec.
Strip one			
Normal	0.41	6.0	6.2
ACh(5.5 μM/liter) + prostigmin (30 μM/liter)	0.32	4.6	3.4
7 min. in Ringer	0.40	6.5	6.0
Strip two			
Normal	0.23	3.6	3.7
Normal, 40 min. later	0.25	3.4	4.5
ACh(5.5 μM/liter) + prostigmin (30 μM/liter)	0.25	1.8	2.4
ACh(5.5 μM/liter) + prostigmin (30 μM/liter)	0.19	2.7	1.7
4 min. in Ringer	0.35	4.5	5.2
Strip three			
Normal	0.36	9.8	7.8
Normal, 1 hr. later	0.34	9.2	9.1
ACh(5.5 μM/liter) + prostigmin (30 μM/liter)	0.18	4.4	2.8
12 min. in Ringer	0.33	8.2	8.7
ACh(5.5 μM/liter) + prostigmin (30 μM/liter)	0.25	4.6	3.1
25 min. in Ringer	0.36	6.9	10.0
ACh(5.5 μM/liter) + prostigmin (30 μM/liter)	0.29	2.6	3.9
Average reduction, *per cent*.....	29	44	59

To evaluate the changes in λ it should be noted that

λ is equal to $\sqrt{\frac{r_m}{r_i + r_e}}$ in which

r_m = resistance × unit length of the surface membrane in ohm cm.
r_i = internal resistance per unit length of the fibers in ohm cm.$^{-1}$
r_e = resistance per unit length of the external fluid in ohm cm.$^{-1}$

Note also the basic constants:

$R_i = \pi a^2 r_i$ = specific resistivity of the myoplasm in ohm cm. (a = radius of fibers in centimeters).
$R_m = 2\pi a r_m$ = transverse resistance of the surface membrane in ohm cm.^{2} (9).
C_m = capacity per unit area of the surface membrane in fd./cm.^{2}.

On the average, the characteristic length with 5.5 μM/liter acetylcholine and 30 μM/liter prostigmin application was reduced about 30 per cent. If we as-

sume that the internal and external resistances are unchanged, this indicates that the membrane resistance r_m must have been reduced even more, because λ is proportional to the square root of r_m.

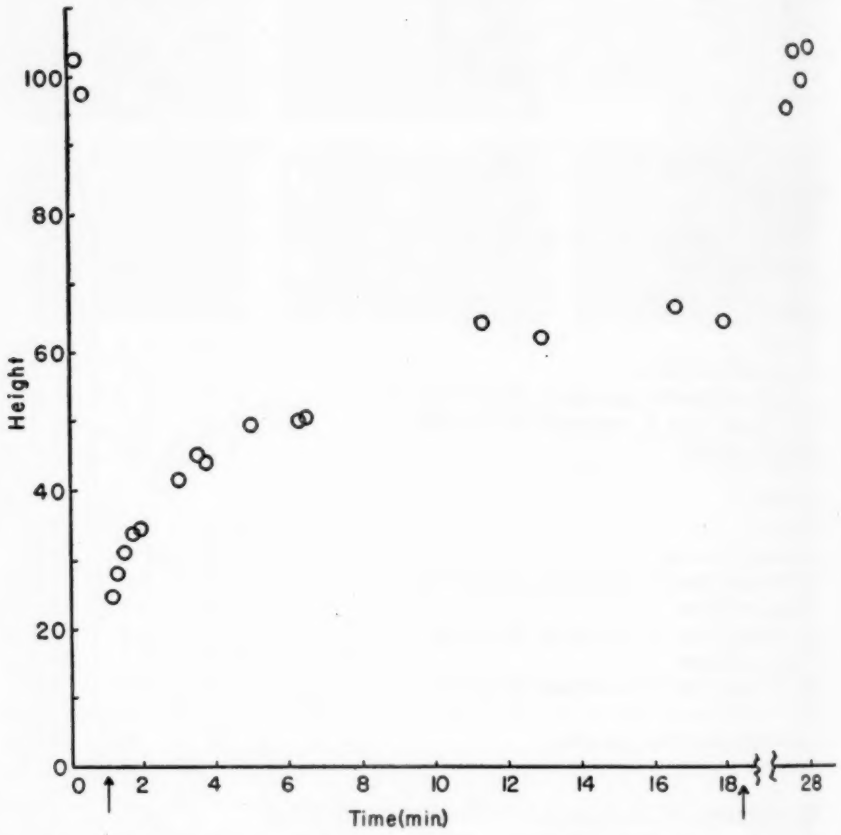

FIG. 3. Height of electrotonic pulse as a function of time. Position of recording electrodes fixed. At the first arrow a drop of 55 μM/liter acetylcholine without prostigmin was applied to the preparation for 20 seconds. The preparation was bathed in Ringer for about 10 minutes starting at the second arrow and afterwards measurements were repeated.

Measurement of the Time Constant of the Membrane (τ_m): There are several ways to evaluate τ_m from such records as are shown in Fig. 1 (9). One method is to measure the time from the beginning of the pulse to the point of half the maximal height; the slope of the plot of this time as a function of interelectrode distance is $\tau_m/2\lambda$ (τ_{m1} in Table I). An alternative method is to fit

one or more points on the experimental curves to the theoretical formula (9) (τ_{m2} in Table I).

The time constant was measured by both methods for the same series of experiments in which the characteristic length was measured (see records in Fig. 1). The results are given in Table I. There was a reversible decrease of the time constant due to acetylcholine application, and the average decrease was about 50 per cent.

Transient Changes after Acetylcholine Application.—In the absence of prostigmin the acetylcholine effect was transient. An example is given in Fig. 3. A drop of 55 μM/liter (1 part in 10^5) acetylcholine was applied for 20 seconds and the changes in the electrotonic potential were recorded at a fixed distance from the polarizing cathode. As a result of reduction of the length constant the electrotonic potential height was reduced by about 70 per cent, measured within 1 minute of the application. Within 14 minutes half recovery had occurred; *i.e.* the effectiveness of acetylcholine was reduced by 50 per cent. Immersion in Ringer for 10 minutes restored the original potential height. In contrast, in the presence of 5.5 μM/liter (1 part in 10^6) acetylcholine but with 30 μM/liter prostigmin added, the potential showed only a small change over the same period (Fig. 4).

The curve of Fig. 3 may well represent the time course of hydrolysis of the small quantity of acetylcholine which remained after the drop was removed. Some sort of adaptation, *i.e.* a loss of effectiveness of the drug may occur in addition, as observed by Thesleff (23) in skeletal muscle.

Values of the Membrane Constants.—The evaluation of the so called basic constants (R_i, R_m, and C_m) is quite difficult for the syncytial system of the heart. By the method of Hodgkin and Rushton (9) r_i and r_m were measured. The specific resistance of the myoplasm (R_i) was assumed to be 100 ohm cm. (value given by Weidmann (24) for kid heart Purkinje fibers). From these figures the radius of the fibers was calculated to be about 9 μ and the membrane resistance R_m to be 280 ohm cm.^{2}. R_m was reduced to 220 ohm cm.^{2} in the presence of 5.5 μM/liter acetylcholine and 30 μM/liter prostigmin. The membrane capacitance in Ringer was about 3.0 μfd./cm.^{2}.

Effect of Acetylcholine on Threshold.—When the electrotonic potentials near the cathode attained a critical height, first partially and then fully conducted impulses arose after varying latent periods. With stimuli well above threshold only fully propagated impulses were seen, the latent period decreasing with increasing stimulus strength. If a drop of acetylcholine (5.5 to 550 μM/liter) in Ringer was applied and then removed after 30 to 60 seconds, the threshold stimulus current strength for conducted impulses was always increased (*cf.* reference 3). At the same time the height of the electrotonic potential at which conduction occurred was considerably reduced. We were unable to determine, however, whether the "firing level" of the muscle fiber had ac-

tually changed. The alteration of the electrotonic potential could be due to a reduction of the length constant as the recording electrode did not lead accurately from the site of the impulse origin. Under present conditions the monophasically recorded action potential was greatly shortened after acetylcholine application. Complete block of conduction, expected according to results with vagal stimulation (14), did not occur in the present experiments

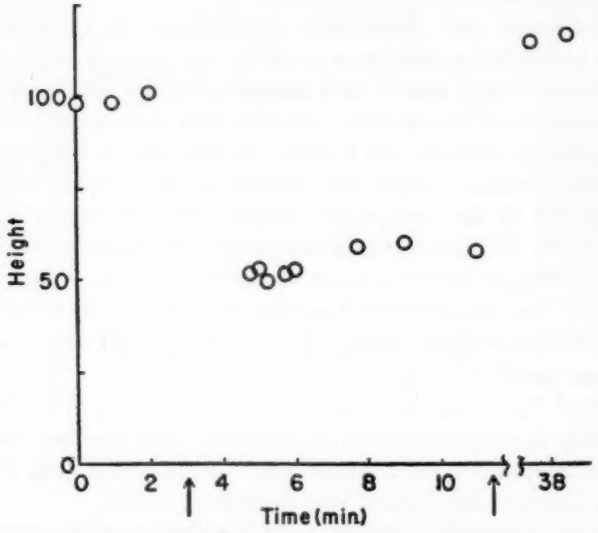

FIG. 4. Height of electrotonic pulse as a function of time. At the first arrow a drop of acetylcholine (5.5 μM/liter) and prostigmin (30 μM/liter) was applied for 30 seconds. The tissue was bathed in Ringer for about 25 minutes starting at the time indicated by the second arrow and afterwards measurements were repeated.

even with the highest concentration of acetylcholine (5.5 mM/liter or 1 in 10^3). Only a reduction of the action potential height was seen.

DISCUSSION

Resistances across membranes can be measured satisfactorily by inserting two microelectrodes into a single muscle fiber, passing current through one and determining the resulting membrane potential changes through the other (6). This method proved too difficult in the present preparation although it has been done on the relatively large Purkinje fibers (24). A simpler approach, similar to that of Araki and Otani (1), consisted of inserting only one electrode and using it simultaneously for recording and for passing current through the cell. Although direct readings of membrane resistance were thus obtained, fluctuations in the resistance of the micropipettes during insertions into the heart muscle cells and during passage of even small currents, reduced

the usefulness of this method. Kahn (15), using the method of Schaefer, Schölmerich, and Haass (21), similar to ours, measured successfully electrical properties of heart muscle strips but at that time no clear-cut conclusions could be reached about the action of acetylcholine on the length and time constants.

The present study has shown that inhibition produced by acetylcholine increases the conductance of the membrane. At the same time the membrane potential was not greatly altered with such effective concentrations as 5.5 μM/liter, because conducted action potentials were not appreciably changed in height. This is not unexpected, since the inhibitory transmitter tends to shift the membrane potential of heart muscle fibers towards the "resting" level (4, 14). Therefore it may be assumed that only certain ions are permitted to flow during inhibition. The K^+ and Cl^- ions, whose equilibrium potential is presumably near the full membrane potential level, are most likely to be involved (for recent results see 3, 4, 5, 14, 17). Earlier evidence (13, 18) has pointed to an increased K^+ liberation during vagal stimulation in mammalian and tortoise hearts and more recently Holland, Dunn, and Greig (11, 12) measured increased rates of K^+ loss in K^+-free solution. In K^+-rich solutions the uptake was reported to be increased. The importance of Cl^- in inhibitory action as suggested by Coombs *et al.* (5) in the spinal motor neuron has also been found recently in inhibitory junctions of sensory nerve cells of crayfish. Although inhibitory action persists in the absence of this ion, the inhibitory equilibrium level is changed if Cl^- is replaced by various other anions (Hagiwara and Edwards, unpublished observations).

In the present discussion a similarity of action between acetylcholine when applied externally and acetylcholine liberated by vagus stimulation has been assumed. A certain amount of caution is necessary here in view of the very high concentrations which are frequently needed to produce the present effects. Both the vagus and acetylcholine effects fluctuate appreciably in different preparations and at different seasons. This makes strict comparisons, unless made on the same preparation, difficult. In many isolated auricle strip preparations in which innervation was preserved conduction of impulses was readily blocked by vagal stimulation while 5.5 mM/liter acetylcholine did not block completely. Concentrations of acetylcholine of 5.5 μM/liter and higher reduced the long duration of the conducted action potentials, an effect which is characteristic of vagus action (4, 10, 14). It is possible that the acetylcholine concentration at the nerve-muscle junctions actually can be quite high during vagal stimulation. This aspect of acetylcholine action was not studied, although it was clear that the length constants and time constants were progressively changed by increased drug concentrations, for instance from 5.5 to 55 μM/liter. If 5.5 mM/liter was applied, it was difficult to obtain electrotonic potentials, indicating that the membrane resistance was decreased much more than in the average values presented here for lower concentrations.

SUMMARY

Membrane characteristics were studied in isolated muscle strands from auricles of frogs using the "square pulse" technique. Changes in the time course and spatial spread of subthreshold electrotonic potentials were measured. If acetylcholine is applied in concentrations which cause slowing or stoppage of the heart beat, the following changes are produced: (*a*) the length constant (λ) of the membrane is reduced, (*b*) the time constant is shortened. The effects are reversible and increase with acetylcholine concentration. The membrane changes caused by acetylcholine diminish with time.

It is concluded that during acetylcholine inhibition, as well as during vagal inhibition, the conductance of the muscle membrane is increased. Appreciable changes in the resting membrane potential need not accompany inhibition.

We wish to thank Mr. R. B. Bosler for his continued valuable technical assistance.

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THE ACTION OF ULTRAVIOLET RADIATION ON YEAST CATALASE*

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(Received for publication, January 27, 1956)

INTRODUCTION

The series of investigations of which the present paper is part has been devoted to establishing as definitively as possible the "state" of the enzyme catalase within the intact yeast cell.

Cheesman and Davies pointed out in their recent review (1) that "it is difficult, despite a wealth of hypothesis, to name a single case in which the physical state of an intracellular protein has been established." The term *state*, as used by Cheesman and Davies and by others (*e.g.* (2)), is rather vague and requires a more precise definition for our present purposes. We shall therefore define the state of an intracellular enzyme as the sum total of the ways in which it differs from some *reference* state, namely that of the same enzyme extracted from the cell into solution. In the case of yeast catalase, we shall accept as the reference state a dilute solution of the beef-liver enzyme, an extract of the yeast enzyme, or (for most purposes) the enzyme within cells treated by various physical or chemical agents which alter the catalase to a state very like that of the reference state (3, 4).

In view of our previous work, we may already make certain statements about the state of intracellular yeast catalase: (*a*) its activity is low, and rises approximately 15-fold following lysis of the cell (the *Euler effect* (3)); (*b*) its optimal substrate concentration is high, decreasing about fivefold following lysis (3); (*c*) its activity has a qualitatively different pH sensitivity than the reference, although in this case it was not possible rigorously to exclude cell permeability considerations as an explanation of these differences (3); (*d*) it is very stable to the effects of heat (3); (*e*) its activity towards its sub-

* This paper was originally presented at a Symposium ("Photochemical effects in biological systems") of the American Association for the Advancement of Science, held in Berkeley, California, December 28, 1954. The authors are most grateful to two of the other participants in this Symposium, Dr. A. D. McLaren and Dr. R. B. Setlow, as well as to Dr. K. J. Laidler and Dr. B. Dunford, for their criticism of aspects of our work. This research has been supported by grants from the National Cancer Institute (Ottawa) and the National Research Council (Ottawa). This is Paper V in the series entitled "The alteration of intracellular enzymes."

strate is characterized by a high temperature-dependence, *i.e.* the activation energy for the catalase–H_2O_2 system diminishes by approximately 5 kcal./mole following lysis (4); (*f*) the activation energy and entropy for its heat destruction are low, rising by approximately 21 kcal. and 70 entropy unit respectively after lysis (4). These findings indicate that its state is one of relatively low specificity, great disorder.

In addition, we have gained information about the process whereby the intracellular catalase changes to its reference state, the process which we have called *enzyme alteration*: (*a*) the ability of a variety of aliphatic compounds to cause enzyme alteration is correlated with their ability to reduce the energy at an aqueous surface (5); (*b*) the activation energy of alteration induced by *n*-butyl alcohol (hereafter butanol) and chloroform was about 22 kcal. (6); (*c*) alteration of catalase was found to be probably not all-or-none per cell (5) but probably to be all-or-none per molecule of catalase (6); (*d*) with optimal concentration of altering agent, the rate of alteration seemed to be of zero order with respect to enzyme concentration, and was actually found to be independent of the concentration of yeast cells, and thus presumably of enzyme concentration (6). All these data were consistent with the hypothesis that alteration of catalase consisted in desorption of the enzyme from some interface at which it is normally adsorbed in a relatively disordered condition within the living cell (the *interfacial hypothesis*).

In view of our earlier work on the effect of ultraviolet radiation (UV) on yeast catalase (3, 4), it seemed advisable to study more closely the kinetics of UV-induced catalase alteration. This study has permitted us to confirm many of the above findings, and to acquire a bit of additional information about the state of this intracellular enzyme.

EXPERIMENTAL

Methods of washing, handling, and preparing the yeast cells were the same as those used in previous studies (4, 5). Cells were suspended in phosphate buffer, pH 7.2, throughout, except when otherwise specified. The lamp used in the irradiations was the same as that described by Aldous and Stewart (7) and by us (8); irradiations were carried out in the cold room or at room temperature, variations never exceeding 1°C. during any run to be described. Our Beckman DU spectrophotometer was calibrated by means of a mercury lamp, and was within the suggested wave length tolerances throughout the range studied (*e.g.* 1 A at 265.2 and 253.6 mμ).

The ribonucleic acid (RNA) was either a commercial preparation from yeast (Nutritional Biochemicals, Cleveland, Ohio), the same deproteinized by the method of Sevag, Lackman, and Smolens (9), or a highly purified pancreatic RNA kindly given us by Dr. A. Marko of the National Research Council, Halifax. In all cases in which RNA was added to the catalase solutions, pH was restored to the desired level by addition of NaOH. Crystalline beef-liver catalase was the same as that used previously (3, 4) and the crystalline ribonuclease was obtained from the Worthington Laboratories (Freehold, New Jersey).

RESULTS

A. Preliminary.—Since we shall be discussing the effect of temperature on at least 4 different reaction rates, we summarize in Table I the principal reactions to be distinguished. Activation energies for reactions 1 to 4 will be differentiated by corresponding subscripts ($\mu_1 \ldots \mu_4$, etc.).

The over-all picture of the effect of polychromatic UV radiation on intracellular yeast catalase may be seen by following curve A in Figs. 1 and 2. The effect of UV may be devided conveniently into 4 periods; *period 1*, during

TABLE I

Reaction Rates upon Which the Effect of Temperature Has Been Studied. Reaction 2 Was Studied in Reference 4

Reaction	Experimental variables	Symbol of μ
1. $2H_2O_2 \rightarrow 2H_2O + O_2$ catalase	UV treatment (or control) at constant temperature, catalase assay at both 5 and 30° after irradiation	μ_1
2. Active catalase $\xrightarrow{\Delta}$ inactive catalase	Cell suspensions heated for varying times at high temperatures, activity then assayed at 30°	μ_2
3. Unaltered catalase $\xrightarrow{h\nu}$ altered catalase	UV irradiation at both 3 and 25°, with subsequent catalase assay at constant temperature (either 5 or 30°)	μ_3
4. Altered catalase $\xrightarrow{h\nu}$ inactive catalase	Same as 3	μ_4

which all the cells become inviable, using as a criterion their inability to form colonies on nutritive medium (see inset, Fig. 1) but during which no change in catalase activity of the suspension occurs; *period 2*, during which an abrupt and dramatic increase in catalase activity (the Euler effect) takes place, to a level, in the case of curve A, seventeen times that of the cells in period 1; *period 3*, during which irradiation produces no change in the maximal catalase activity of the suspension, and *period 4*, during which the enzyme is irreversibly destroyed by the UV. There is no correspondence between the numbering of these periods and that of the reactions in Table I.

The yeast suspensions used in these studies were divided into several categories (A to E) as regards temperature of irradiation, of storage (between irradiation and assay), and of assay of catalase activity. The physical meanings of the activation energies obtained by comparing these various categories

are summarized in Table II, to which reference should be made during our treatment of data concerning these categories.

B. Periods 1 and 2.—In our previous experiments, the UV-induced Euler effect (3) had been found to be accompanied by a decrease in μ_1 (4); in other words, UV not only greatly increased the catalase activity of a yeast suspension but at the same time decreased the temperature dependence of its

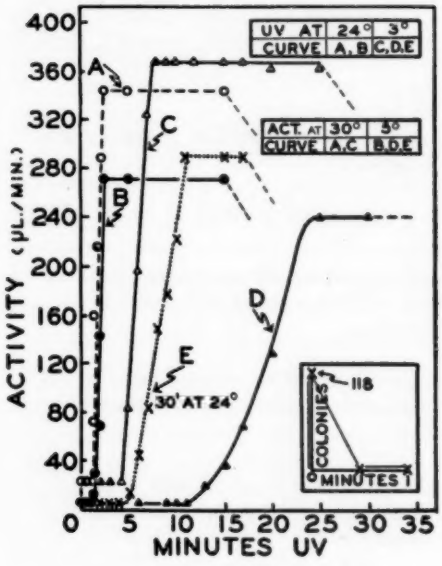

FIG. 1

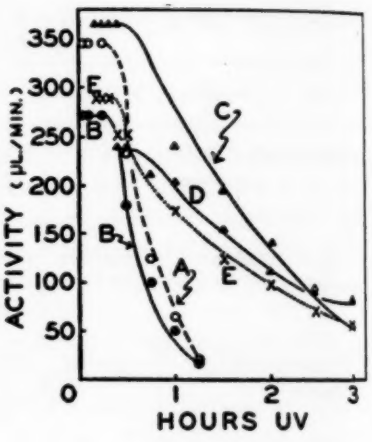

FIG. 2

FIG. 1. Effect of temperature of irradiation, of storage, and of assay on the rate of alteration of catalase in cells of a 0.02 per cent yeast suspension. Inset, effect of irradiation on viability of the suspension irradiated at room temperature.

FIG. 2. Effect of temperature on rate of inactivation of yeast catalase and on duration of the protection effect; these curves are continuations of those of Fig. 1.

activity. In Fig. 1, curves A and B represent changes in catalase activity of a yeast suspension irradiated at constant temperature (24°C.) and assayed subsequently at 30° (A) and 5° (B). Thus, comparison of points along these curves will show the effect of temperature on the enzyme-substrate system and changes in this effect as a consequence of prior irradiation. Inspection of the curves shows that initially (period 1) there was a several-fold decrease in activity as the temperature of assay was lowered from 30° (A) to 5° (B); however, when maximal activity was reached (period 3) it will be noted that the catalase activity became much less temperature-dependent; *i.e.*, the μ_1 had decreased. When one substitutes the individual activities. of which these curves are

TABLE II

Categories of Yeast Suspensions, with Respect to Temperatures of Irradiation, Storage, and Assay, and Physical Significance of Activation Energies Calculated from Their Comparison

Reaction designations refer to Table I. The temperature of storage is that at which the suspension was kept after irradiation and before assay.

Category	Temperature of irradiation	Temperature of storage	Temperature of assay	Post-UV heat (> 5°)
		A		
	°C.	°C.	°C.	
A	24–25	24–25	30	Present
B	24–25	24–25	5	Present
C	3	24–25	30	Present
D	3	0	5	Absent
E	3	24–25	5	Present

B

Comparison of rates of categories	Reaction studied	Temperatures used in Arrhenius equation (°C.) + 273	Interpretation of physical meaning of μ values obtained by use of indicated rates of alteration and temperatures
A and B	1	5, 30	μ_1 will represent mean of all μ_1 values during alteration process; should be intermediate between initial (unaltered) and final (altered) levels, since rates were taken from slopes of the alteration curves
A and C	3	3, 25	μ_3 will not include energy required to activate post-UV reaction, since C was subject to higher temperatures not included in calculation of μ_3
B and D	3	3, 25	μ_2 will include the post-UV component, since D was not subject to temperature higher than 5°. μ_4 will be over-all energy of activation of alteration
B and E	3	3, 25	Same as A and C above
C and D	1	5, 30	Same as A and B above
C and E	1	5, 30	Same as A and B above
D and E	? (part of 3)	3, 25	μ_3 probably represents energy of activation of the post-UV reaction

composed, into the Arrhenius equation, one determines the μ_1 at any stage of the irradiation. One can thus plot the μ_1 values *versus* time of irradiation, as in Fig. 3, Curve AB, which provides, in effect, a measure of the rate of catalase alteration since it illustrates the rate of change of μ_1 during the irradiation.

In the experiment represented in Fig. 3, the μ_1 decreased from its initial value of approximately 8 kcal. to a final value of approximately 2 kcal. A most unexpected finding was that before dropping to the final level characteristic of the altered enzyme, the μ_1 rose at the onset of period 2 to a level well above that of the unaltered enzyme, in the case of the suspension represented in

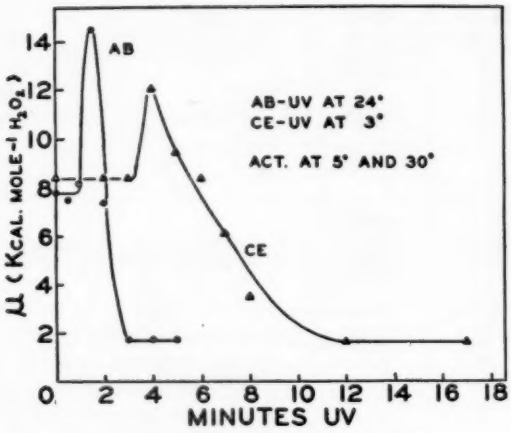

FIG. 3. Change in μ_1 with time of irradiation.

curve AB to over 14 kcal. at 1.5 minutes' irradiation. At this time one notes in Fig. 1 that the yeast suspension irradiated at 24° demonstrated an approximately fourfold increase in catalase activity when this was assayed at 30° (curve A, 4th point), but an activity which had not quite doubled when assayed at 5° (curve B) thus accounting for the unusually high activation energy at the onset of period 2. Since this phenomenon has been found in most experiments (and, indeed, was of greater duration when irradiation was carried out at lower temperature), it appears that this period may be divided into 2 *a*, the first phase of alteration during which the μ_1 rises to a level higher than that of the unaltered enzyme, and 2 *b*, the subsequent phase during which the μ_1 rapidly drops to the low level characteristic of the altered enzyme.

Curves C, D, and E (Fig. 1) represent the course of change of catalase activity of a single suspension irradiated at 3°C.; its activity was thereafter assayed either at 30° (C) or 5° (D, E). The aliquots represented in curve D were stored on ice between irradiation and assay whereas those in curve E were brought to room temperature for 30 minutes and thereafter cooled to 5° for

activity assay. In this way, we have determined to what extent heat may act on this system *after* (we shall call this post-UV heat) as well as *during* the irradiation. Change in μ_1 during the irradiation at lower temperature is shown in Curve CE (Fig. 3), which was qualitatively much like AB, except that the onset of period 2 with its change in μ_1 was delayed considerably.

Data on μ_1 are summarized in Table III. In line 1, the mean initial activities at the two temperatures were compared to yield an average μ_1 before alteration. In lines 2 and 3 the maximal levels (of period 3) were compared to yield an average μ_1 after alteration. Since the difference between μ_1 of lines 2 and 3

TABLE III

Experimental Activation Energies for Reaction 1 (μ_1) Showing Change in This Constant during Irradiation

Standard deviations are shown. In lines 1 to 5, the μ_1 was determined by comparison of activities at two temperatures either before (1) or after (2 to 5) maximal UV-induced Euler effect. In lines 6 to 8, μ_1 was determined by comparison of slopes of alteration curves, such as those of Fig. 1 (period 2).

	Categories compared	n	μ
			kcal./mole
1	A and B C and D, E	12	8.2 ± 0.4
2	A and B	7	1.5 ± 0.3
3	C and E	4	2.3 ± 0.8
4	Average		1.9
5	C and D	5	3.0 ± 0.8
6	A and B	5	4.1 ± 1.9
7	C and E	4	4.6 ± 1.6
8	C and D	4	10.2 ± 1.9

was not significant, these data have been averaged in line 4. Thus, during the irradiation, μ_1 dropped from 8.2 to 1.9 kcal., a somewhat greater decrease than that previously reported (4). The μ_1 determined by comparison of categories C and D (line 5) is significantly different from that of line 2, and the meaning of this finding will be discussed below. In lines 6 to 8, μ_1 values were determined from the slopes of the alteration curves, in which the activity of the UV-treated yeast aliquots was assayed at both 5 and 30°C. Activation energies determined in this way are simply evenly weighted averages of all μ_1 values during the UV-induced alteration process (see Table II) and, as expected, were, in the case of lines 6 and 7, intermediate in value between those of period 1 and of period 3. The physical meaning of the μ_1 of line 8 must be exactly the same as that of lines 6 and 7; the very high μ_1 recorded here indicates that when the effects of post-UV heat have been excluded, as in category D, greater weight is given in the calculation of μ_1 to the initial phase of period 2 (2 *a*) which was

characterized by an extremely high μ_1. Indeed, it is obvious from comparison of curves C and D (Fig. 1) that period 2 *a* has a very considerable duration due both to the delay in onset of period 2 in category D (no post-UV heat) and to the very low rate of alteration of catalase in suspensions of this category.

Let us now consider the effect of temperature on the *process of alteration* itself (reaction 3) rather than on the enzyme-substrate system (reaction 1). To do this, we shall compare the activity of yeast suspensions irradiated at two different temperatures but subsequently assayed at constant temperature. Comparison of curves A and C (Fig. 1), representing cells which were irradiated at 24 and 3° respectively but assayed at 30° after irradiation, illustrates the

TABLE IV

Effect of Temperature on the UV-Induced Alteration Process

Velocity of alteration is given by the relation $v = (A_m - A_0)/t$, using symbols defined below. Standard deviations shown. The difference between any two of the mean velocities of column 7 is significant, at least at the 5 per cent level.

1	2	3	4	5	6	7
Category	n	Duration of lag 1	Initial activity (A_0)	Final (maximum) activity (A_m)	Duration of alteration (t)	Velocity (v) of alteration
		min.	*µl./min.*	*µl./min.*	*min.*	*µl./min.*^{2}
A	13	1.0 ± 0.1	23 ± 5	323 ± 6	1.5 ± 0.5	230 ± 64
B	6	1.0 ± 0.0	8 ± 2	278 ± 32	2.0 ± 0.9	163 ± 65
C	11	4.7 ± 2.5	23 ± 6	316 ± 36	7 ± 4	52 ± 24
D	7	12.0 ± 4.0	9 ± 1	190 ± 42	24 ± 20	8 ± 5
E	4	6.0 ± 3.0	7 ± 2	236 ± 57	10 ± 5	27 ± 13

large temperature dependence of photochemical alteration. The big differences in duration of both period 1 and period 2 show that heat applied to the cells after irradiation (as in category C) was not able to reproduce the accelerating action of heat applied during the irradiation. On the other hand, comparison of curves D and E, in which cells were irradiated (5°) and assayed (30°) at the same temperatures, but in which category E was brought to room temperature between irradiation and assay, shows that the post-UV heat brought to bear on category E considerably accelerated both the time of onset and the rate of the reaction of alteration. Thus part of the action of heat on alteration was to accelerate a reaction which could take place *after* the primary photochemical reaction had ceased.

Our data on the effect of heat on rates of alteration are contained in Table IV. The rates of alteration, having here the dimensions of µl. O_2/min.^{2}, (*i.e.*, acceleration of the rate of O_2 production from enzyme-catalyzed H_2O_2 breakdown) were determined either as in column 7 (Table IV) by dividing the change in rates of catalatic activity by the duration of irradiation required to

achieve the change, or directly from the slopes of the alteration curves, such as those of Fig. 1.

Data on μ_3 are summarized in Table V. The over-all activation energy for photochemical alteration of catalase is, we consider, represented by the value of line 5, determined from the velocities of alteration of categories B and D, since only this μ_3 value will include a component for the effect of heat acting after the irradiation. The mean value thus obtained, 23.6 kcal., happens to be quite close to those reported for the temperature-dependence of alteration by butanol and chloroform (20 to 24 kcal., reference (6)) and to that incorrectly predicted from the difference between the μ_2 values of altered and

TABLE V

Activation Energies for Reaction 3

Activation energies were determined by comparing either the velocities of alteration (slopes of curves in Fig. 1), or the reciprocals of the duration of lag period 1. Standard deviations shown.

Categories compared	n	μ determined from	μ_3
			kcal./mole
1. A and C	7	Slopes	13.6 ± 3.4
2. A and C	8	Lag	12.1 ± 3.7
3. B and E	2	Slopes	17.7
4. B and E	2	Lag	11.7
5. B and D	4	Slopes	23.6 ± 1.6
6. B and D	4	Lag	17.6 ± 1.2
7. D and E	2	Slopes	7.6
8. μ_3 (BD) − μ_3 (BE) = 23.6 − 17.7 = 5.9			

unaltered yeast catalases (4). The lower values of μ_3 of lines 1 and 3 (Table V) are due to the fact that post-UV heat (which was acting in all four of these categories) was excluded from calculation of the activation energy, as will be noted from Table II.

We may speculatively estimate the activation energy of the reaction(s) accelerated by heat after the primary photochemical reaction has ceased. One way of doing this is by calculating μ_3 by substituting into the Arrhenius equation rates of alteration of categories D and E, differing only in the temperatures at which they were stored for a 30 minute period following irradiation. As noted in line 7 (Table V), this yields a value of 7.6 kcal. Another way of estimating this constant is by subtracting from the over-all activation energy (line 5), calculation of which included the post-UV heat-sensitive process, the value from which this process was excluded (line 3). As noted in line 8, this method gives a value of 5.9 kcal., permitting an average estimate of 6.8 kcal. for the activation of this hypothetical reaction.

If one substitutes into the Arrhenius equation not the velocities of alteration but

rather the reciprocal of the duration of period 1, one obtains the values of μ_3 represented in lines 2, 4, and 6 of Table V. One may speculate that the duration of this lag would be inversely related to the rate of whatever reaction must occur before catalase alteration can take place. Such a preliminary reaction would also be accelerated by heat applied after the irradiation (Fig. 1) and its over-all activation energy would be the 17.6 kcal. of line 6.

The effect of cell concentration on rate of alteration is shown in Fig. 4. As expected, the greater the density of the suspension the lower the velocity of alteration.

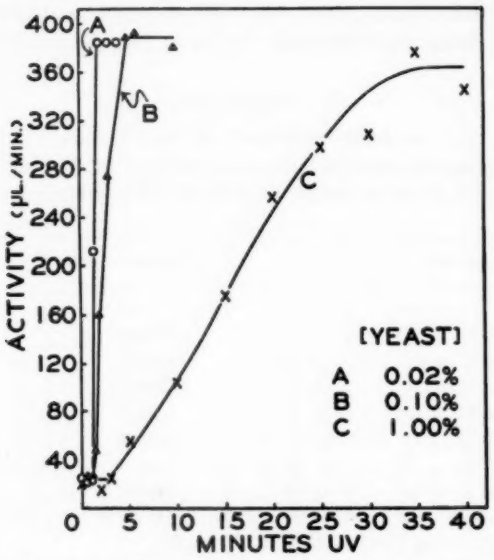

FIG. 4. Effect of concentration of yeast cells on duration of period 1 and on rate of alteration.

In the case of the action of chemical altering agents (6), where shielding cannot occur, the rate was independent of cell concentration.

It is interesting that the alteration curves of period 2 (Figs. 1 and 4) are best described as linear with time, or possibly S-shaped (autocatalytic); none was the typical first-order type curve usually associated with photoinactivation phenomena (see Section D below).

C. Period 3.—A most curious phenomenon discovered in the course of this research was the existence of period 3, the rather long interval following attainment of maximal catalase activity during which further irradiation of the suspension led to no detectable change either in the activity or in the μ_1 (viz. Figs. 1 and 2); we shall call this phenomenon the *protection effect*. In view of the large amount of ribonucleic acid (RNA) in yeast and of the well known loss of nucleotide material from UV-irradiated yeast (10), we adopted the hypothesis

that the protection effect was caused by a simple physical screening, or shielding, of enzyme by cytoplasmic RNA; only after a critical amount of RNA (or at least material absorbing heavily in the spectral region of maximum output of our source, 85 per cent of which was at the 2537 A mercury resonance line) had been lost from the cell, was the incident energy able to inactivate the enzyme.

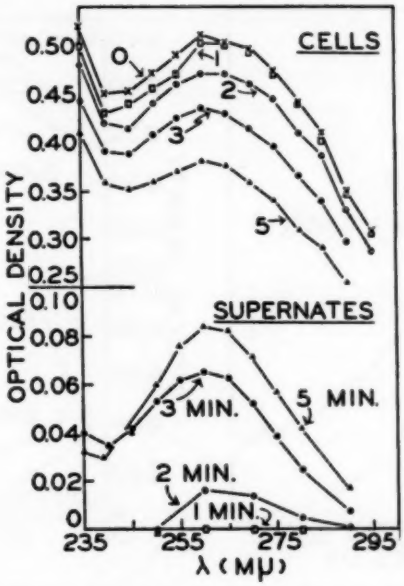

FIG. 5. Absorption spectra of cells irradiated for varying times and of their supernates. 0.1 per cent yeast cells were irradiated and after centrifugation were resuspended to original volume for spectrophotometry. No change in absorbance of either cells or supernates was noted after 5 minutes' irradiation; compare curve B (Fig. 4) for corresponding catalase activities of suspension of this concentration.

If this hypothesis were correct, there should be a simple correspondence in time between loss from the cell of material absorbing in the 260 mμ region and onset of period 4, the photoinactivation of catalase. Changes in the absorption spectra of irradiated cells and of supernates of irradiated cells following centrifugation are shown in Fig. 5. It will be remarked that the absorption at 260 mμ decreased in the cell as it increased in the supernate, showing progressive loss of nucleic acid, or nucleotide-containing material or of the nitrogenous components thereof during the course of the irradiation. However, maximal loss of absorbing material to the external medium was found to occur after about 5 minutes' irradiation at 25° C., whereafter further irradiation caused neither further loss of optical density of the cells nor further increase in

optical density of the supernate. Since the onset of period 4 was never observed until after approximately 20 to 25 minutes' irradiation, it follows that the protection observed during period 3 cannot be due to screening by optically dense material; our hypothesis was thus incorrect.

In view of the important work of McLaren (11), demonstrating that incorporation of chromophores into model peptides stabilized these compounds

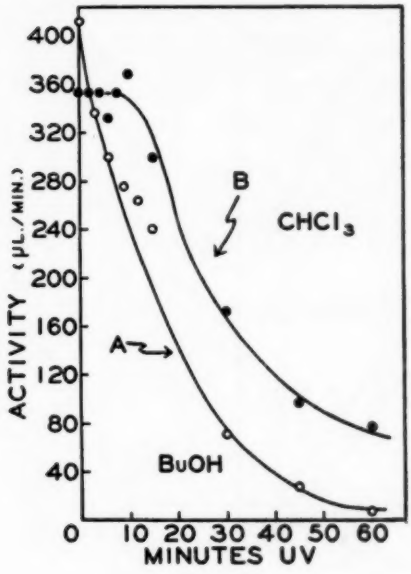

FIG. 6

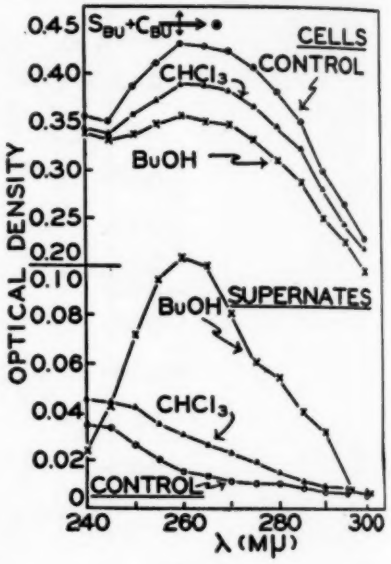

FIG. 7

FIG. 6. Effect of irradiation on catalase activity of 0.02 per cent suspensions previously treated in usual way with butanol (A) and chloroform (B).

FIG. 7. Absorption spectra of unirradiated yeast cells, and of their supernates, before and after treatment with chloroform or butanol. 0.9 per cent suspensions for treatment were made up to 0.1 per cent (after centrifugation) for spectrophotometry.

against UV photolysis, we next adopted the hypothesis that within the cell catalase and RNA existed in some sort of complex which stabilized the former against photochemical inactivation, but that prolonged irradiation split this complex, thus permitting destruction of the enzyme to proceed. Indirect test of this hypothesis has been possible.

It was observed that our two favorite chemical altering agents, chloroform and butanol, differed in their mode of action on catalase, since the latter deprived the altered catalase of the protection effect, whereas the former altered the catalase without loss of this effect. A typical experiment is shown in Fig. 6. The action of the two chemical altering agents on the UV-absorption of treated yeast cells is shown in Fig. 7. The upper set of curves shows that butanol causes

a much greater loss from the cell of material absorbing at 260 mμ than does chloroform, and the lower set shows that a considerable amount of material absorbing specifically at 260 mμ has been liberated into the surrounding medium as a result of butanol treatment but not of chloroform treatment. These data are thus consistent with any hypothesis ascribing the existence of period 3 to the presence within the intact and irradiated cells of material with the spectral properties of nucleic acid, or of its nitrogenous bases. Since simple, physical screening had already been eliminated as an explanation of the pro-

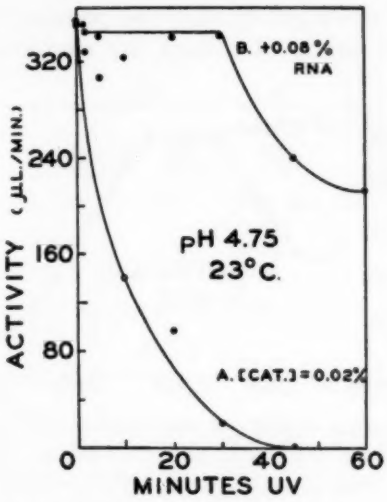

FIG. 8. Effect of RNA on photoinactivation of crystalline liver catalase. Intensity of irradiation approximately one-sixth that of all other experiments to exaggerate the duration of the protection effect. All solutions were diluted suitably for activity assay after irradiation.

tection effect, these data suggested the existence of some form of intracellular interaction between catalase and a chromophore-containing compound.

We then attempted to set up an *in vitro* model exhibiting the protection effect. We found that crude or protein-free yeast RNA, or purified pancreatic RNA could, when mixed with crystalline liver catalase prior to irradiation, protect the enzyme against any appreciable inactivation during the first few minutes of the irradiation. A rather extreme experiment is illustrated in Fig. 8, where mixing and irradiation were carried out at pH 4.75, *i.e.* on the acid side of the isoelectric point of this protein (5.8), where the net charge on the protein moiety would be positive. That interaction with the RNA did actually occur at this pH was shown by the visible turbidity which developed upon mixing the reactants at higher concentration than that used in the irradiation experiments. It will be noted that the RNA–enzyme mixture was completely resistant to UV

irradiation for 30 minutes, during which period the activity of the irradiated catalase control was virtually abolished. It is interesting that, as shown in Fig. 8, the catalase–RNA complex had the same initial activity as the catalase alone, if care was taken to adjust pH of both solutions to the same value.

When similar experiments, with identical concentrations of reactants, were carried out at pH 7.2, *i.e.* on the alkaline side of the isoelectric point where interaction between the negatively charged apoenzyme and the phosphoric acid residues of the RNA might not be expected to occur (and where visible turbidity was not demonstrable), the protection effect was also found. Protection was also afforded at either pH by digestion products of yeast RNA from ribonuclease action, as well as by adenine, but not, curiously, by adenosine-

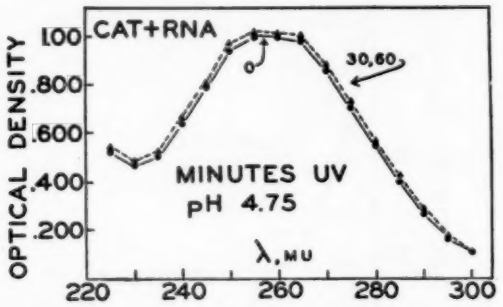

FIG. 9. Absorption spectra of 0.02 per cent catalase–0.08 per cent RNA mixture irradiated for varying times. Irradiation caused no change in spectrum, even when mixture was diluted to bring absorption peak within range of greater sensitivity of the instrument.

triphosphate. The protection effect in the presence of RNA was not diminished by the presence of 10 per cent urea during irradiation. Ovalbumin did not afford protection.

That shielding is inadequate as an explanation of the protection effect is confirmed by the experiment shown in Fig. 9, where absorption spectra for the catalase–RNA mixtures are shown when determined after 0, 30, and 60 minutes' irradiation. The fact that there was no change in absorbance to correspond with the observed inactivation at 60 minutes shows that simple absorption of incident radiant energy by RNA chromophores cannot account for the protection effect in this *in vitro* model. Changes in the absorption spectrum of the irradiated catalase control are shown in Fig. 10; specific absorption at 280 mμ diminishes progressively as non-specific absorption increases, an effect reported for other proteins by Becker and Szendro (12) and by Gates (13). The relatively higher resistance of nucleic acid (than of protein) to UV-induced spectral changes has also been reported from Giese's laboratory (14).

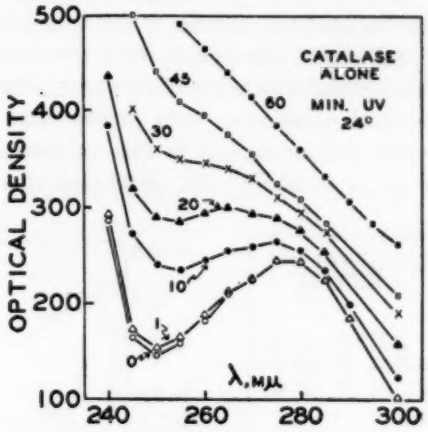

FIG. 10. Absorption spectra of catalase before and after varying periods of irradiation.

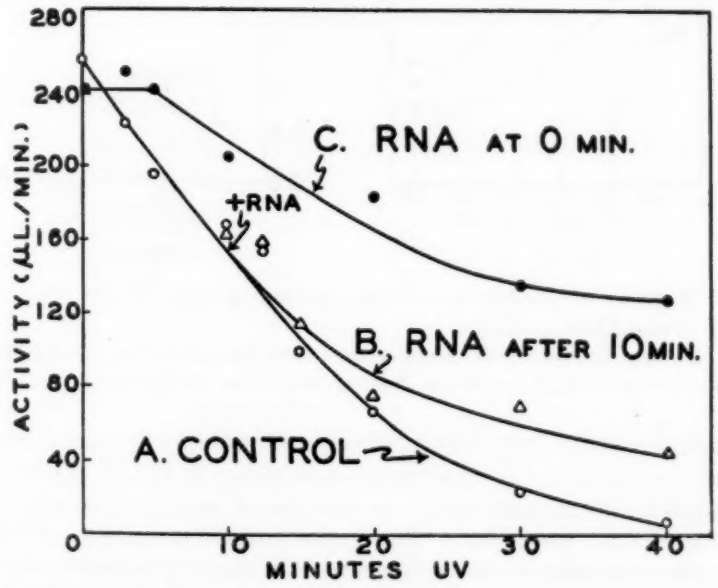

FIG. 11. Effect of 10 minutes' preirradiation of catalase on protection effect (curve B). Solutions buffered at pH 7.2; final RNA concentration was 0.16 per cent, and catalase 0.02 per cent during irradiation. Mixtures diluted for activity assay.

The onset of the photochemical inactivation of catalase might then be due to structural modification of either protein, of RNA, or of both. To determine which of this trio of possibilities was correct, RNA solutions were preirradiated for 10 minutes and then mixed with unirradiated protein before continuing the irradiation; the reciprocal experiment with preirradiated protein and unirradiated RNA was also performed, and in this manner the component first affected by the UV so as to abolish the protection effect was determined.

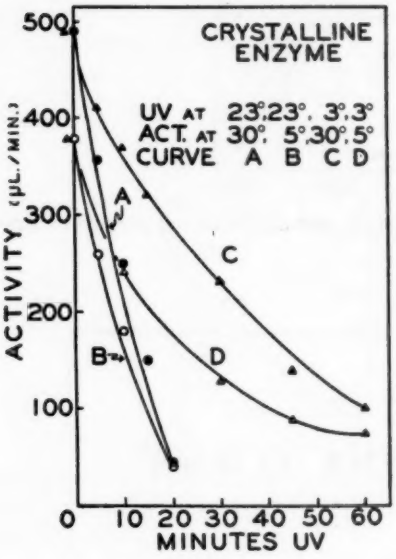

FIG. 12

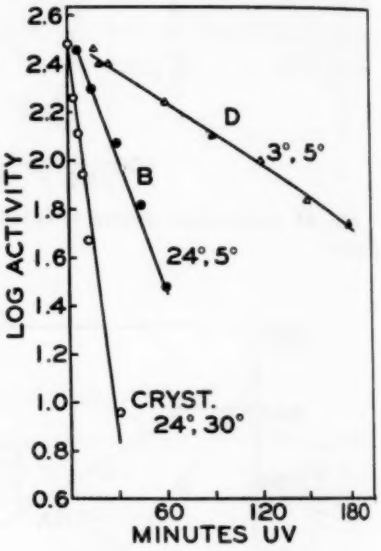

FIG. 13

FIG. 12. Effect of temperature of irradiation and of assay on rate of photoinactivation of crystalline catalase.

FIG. 13. Sample logarithmic inactivation curves for crystalline and intracellular catalases. Temperatures shown are those of irradiation and of assay, respectively.

Preirradiation of the RNA led to no change in its ability to cause subsequent protection of catalase. On the other hand, as shown in Fig. 11, preirradiation of catalase for 10 minutes resulted in abolition of the protection effect when unirradiated RNA was added at that time. A series of experiments was undertaken to determine the minimum duration of UV required to abolish protection, and it was found that, under the conditions of protein concentration and intensity of UV used in the experiments illustrated by Fig. 11, an irradiation of 2 minutes was sufficient to eliminate the protection effect, whereas protein irradiated for but 1 minute behaved as the unirradiated control when subsequently mixed with RNA.

Our data strongly support the hypothesis that the protection effect is due

to stabilization of the intracellular catalase by association with the nitrogenous component of RNA in some as yet unknown manner; as the irradiation proceeds, the catalase becomes modified so as to eliminate the association or to render it no longer effective in protecting the protein from UV-induced inactivation.

TABLE VI

Rates and Activation Energies for Reaction 4

Rates were determined from the slopes of the logarithmic inactivation curves (dimensions: $hrs.^{-1}$). Standard deviations shown.

Experiment	Curve A	Curve B	Curve C	Curve D	Curve E
			A. Rates of inactivation		
1	1.00	1.12	0.26	0.24	0.25
2	1.24	1.12	0.10	0.12	0.12
3	1.66	1.44	0.20	0.20	
4	1.66	1.30	0.13	0.13	
Average.....	1.39	1.25	0.17	0.17	0.19

B. Mean μ_4 values (*kcal./mole*)

Line	Curves compared	n	Temperature of irradiation	Temperature of assay	μ_4
			°C.	°C.	
5	A and C	4	3 and 25	30	16.2 ± 3.4
6	B and D	4	" "	5	16.1 ± 3.5
7	Average		" "		16.2 ± 3.4
8	B and E	2	" "	5	14.6
9	Crystalline catalase	2	" "	30	9.9
10	Crystalline catalase	2	" "	5	10.3
11	Average.....				10.1 ± 0.6
	Crystalline catalase, solid* (approximate mean).....				1.4

* These data from Setlow and Doyle (23), temperature range from − 83 to + 91°C., range of activation energies (calculated by comparing pairs of rates) from 0.9-1.6 kcal.

On the other hand, simple screening is sufficient to account for the differences between rates of inactivation of protein alone and of protein plus RNA demonstrated in Figs. 8 and 11.

Period 4.—The inactivation of intracellular yeast catalase during period 4 is quite similar to that reported for a variety of other proteins irradiated *in vitro*. Fig. 2 shows inactivation curves for the yeast enzyme irradiated and assayed at different temperatures, Fig. 12 the same variables for the crystalline

enzyme *in vitro*, and Fig. 13 that the inactivation of both categories of catalase is a pseudo-first-order process. That rate constants for UV-induced inactivation are not truly first order was shown by McLaren (15) and confirmed by Labeyrie and Shugar (16); that this must be true is apparent from the dependence of these constants on the intensity of the radiation and on the concentration of protein, etc.

Despite the classical demonstration of post-UV heat effects in protein "denaturation" (Bovie (17)), our data, summarized in Table VI, provided little evidence for such effects in the system studied here. In part *A*, the similarity of rates of inactivation of categories C and D shows that post-UV heat had no

TABLE VII

Values of μ_1 during UV-Induced Inactivation of Yeast Catalase

Data from experiment represented in Fig. 2.

Categories A and B		Categories C and E	
(UV at 25°, assay at 5 and 30°)		(UV at 3°, assay at 5 and 30°)	
Duration UV	μ_1	Duration UV	μ
min.	*kcal./mole*	*min.*	*kcal./mole*
3	1.6	17	1.7
15	1.6	25	2.5
30	1.4	60	1.6
45	1.5	90	3.0
60	1.6	120	0.8
75	0	150	2.1
		180	0

measurable effect. On the other hand, comparison of categories A with C and B with D shows that the photochemical inactivation was quite temperature-sensitive, being characterized by a μ_4 of approximately 16 kcal. (part *B*, Table VI). This finding is quite different from that of Gates (18) who irradiated pepsin at 8 and 22° and found a Q_{10} of close to unity ($\mu \cong 0$).

Finally, Table VII illustrates the constancy of μ_1 throughout the photoinactivation of yeast catalase. Only after extremely prolonged irradiation did the μ_1 change, dropping to vanishingly small, or negative (*e.g.*, compare final points, curves C and D, Fig. 2) values.

DISCUSSION

A. Periods 1 and 2.—In terms of our interfacial hypothesis, the events occurring during period 1 could be either a modification of the surface at which catalase is adsorbed within the cell, or a release from the irradiated protoplasm of an endogenous altering agent of greater surface activity than the protein,

resulting in displacement of the latter from the interface in question. It is known that UV causes a release of a variety of substances from irradiated yeast (Loofbourow *et al.* (10)), and Novick and Szilard (19) have hypothesized the release of a cytotoxic agent in UV-treated bacteria.

While we cannot exclude either hypothesis at present, the acceleration of the rate of photochemical alteration by post-UV heat indicates that, if an endogenous altering agent were released by the irradiation, its surface activity must have quite different temperature characteristics than those of exogenous altering agents, like butanol (6). The first of this alternative of hypotheses, *i.e.* photochemical modification of the intracellular surface, which has the additional advantage of being heuristic, is consistent with the following model of UV-induced alteration:

$$C - S \underset{k_{-1}}{\overset{h\nu}{\rightleftharpoons}} C - S^* \qquad 1\,a$$

$$C - S^* \xrightarrow{k_2} C + S \qquad 2\,a$$

in which $C - S$ represents catalase, C, adsorbed at surface S, and $C - S^*$ a metastable intermediate.

The reversibility of reaction 1 *a* is indicated by the presence of the post-UV heat effect. It is also indicated by the finding that the alteration of the catalase of category D did not reach completion, but attained an equilibrium position during period 3 in which some of the intracellular catalase remained unaltered. This was evident from the data of Table IV (column 5) showing a lower maximal activity for category D than for either B or E (assayed also at 5°), and from those of Table III, line 5, showing a μ_1 for categories C and D of 3 kcal., which was significantly higher than the μ_1 for categories A and B. It is clear from this that cells irradiated at 3° and not subject to temperatures higher than 5° during or before assay never demonstrated complete alteration of their catalase, indicating the existence of an equilibrium position which, in the case of cells subjected to higher temperatures at any time before assay, was shifted in the direction of more complete alteration.

Since the primary photochemical reaction may be considered to be temperature-independent, the acceleration of the rate of alteration at increased temperature must be determined by the extent to which k_2 is more accelerated than k_{-1}. Our data, alluded to immediately above, suggest that k_{-1} is relatively less temperature-sensitive than k_2: at low temperatures where an equilibrium between unaltered and altered catalases exists, k_2 was only slightly higher than k_{-1}, but as the temperature was raised either during or after the irradiation, k_2 was relatively more accelerated and the equilibrium shifted in the direction of alteration. According to this model, step 2 *a* is rate-limiting in the over-all reaction of photochemical alteration.

Independent evidence which may be taken to support our model comes in a personal communication from our former collaborator, M. J. Fraser, presently working in the laboratory of J. H. Schulman on the effect of ionizing radiations on yeast catalase. Making certain reasonable assumptions, he calculated target molecular weights for alteration (period 2) and for inactivation (period 4) of 3 to 4 $\times$ 10^6 and 2 $\times$ 10^5, respectively. The latter figure is quite close to the accepted molecular weight of liver catalase (225,000 to 250,000), whereas the former suggests that the site modified during alteration is, or contains, RNA. If, as both Fraser and we believe, the alteration process follows upon a modification of a RNA-containing surface (microsome?), it should be possible to adduce evidence bearing on this hypothesis from a study of the action spectrum of the photochemical alteration.

That the site of absorption and action of the radiant energy during alteration is the catalase itself seems most unlikely in view of the decreased entropy level of the altered enzyme (4).

The mean, over-all activation energy for UV-induced alteration was 24 kcal., a value quite close to those reported for alteration by butanol and chloroform (20 to 24 kcal.). While this might at first glance suggest a common rate-limiting step, this cannot be the case, since the rate of alteration induced by these agents was not accelerated by heat *following* treatment. A satisfactory model of alteration induced by butanol (BuOH) is:

$$C - S + \text{BuOH} \xrightarrow{k_1} C - S - \text{BuOH} \qquad 1b$$

$$C - S - \text{BuOH} \xrightarrow{k_2} C + S - \text{BuOH}, \qquad 2b$$

in which the symbols have their previous meaning. The absence of posttreatment heat effect indicates that reaction 1 *b* was rate-limiting in this case. There was no evidence of reversibility of any of these reactions; another difference from photochemical alteration was the inability of prolonged treatment with butanol at 3° (in excess of 24 hours) to induce alteration.

Experimentally obtained activation energies for alteration by both UV and chemical agents were close to the value predicted by one of the present authors from a consideration of the differences in μ_2 of unaltered and altered yeast catalases (4). However, in view of the fact that these data actually indicated that the unaltered enzyme was *higher* on the energy scale (by 21 kcal.) than the altered (*i.e.*, bonds totalling 21 kcal. strength were believed to be formed during alteration) no prediction whatever of minimum activation energy for alteration ought legitimately to have been made. This prediction is hereby retroactively retracted.

B. Period 3.—We have presented evidence indicating that the cause of the protection effect, *i.e.* the rather long period during which irradiation of the yeast suspensions produced no inactivation of their maximally active catalase,

was the stabilization of this intracellular enzyme by means of a complex with RNA, or at least with its nitrogenous components. Cheesman (20) has demonstrated protein–nucleic acid interaction utilizing protein films and nucleic acid injected into the aqueous substratum; he attributes the interaction to hydrogen bonding, since it was abolished by low concentrations of urea. On the other hand, the presence of 10 per cent urea did not diminish the protection effect in our experiments. In our laboratory, we have not been able to confirm Cheesman's observations that small concentrations of RNA and DNA exerted a marked expanding effect on isoelectric protein films. However, we have obtained evidence (Kaplan, unpublished data) of interaction between films of catalase, at both pH 7.2 and 4.6, and very low concentrations of RNA and adenine, in that such films become very resistant to the peptide bond photolysis previously demonstrated (21, 8). Since McLaren has shown (11) that phenyl groups stabilize keto-imino ("peptide") linkages against photolysis, it would appear that foreign chromophore groups, even when loosely linked to protein, act to stabilize, rather than to sensitize, the latter molecules. The protein moiety of tobacco mosaic virus also seems to be protected by its nucleic acid from changes in its physical properties induced by UV irradiation (11, p. 155).

We were able to demonstrate *in vitro* a protection of catalase by means of ribonuclease-produced RNA degradation products and by adenine. Hence, it is obviously not possible to inculpate polymerized RNA as the intracellular agent necessarily responsible for this effect, although the high concentration of this substance in yeast makes this seem not improbable. The protection effect was not completely non-specific; ATP and ovalbumin were without action.

C. Period 4.—The photoinactivation of both intracellular yeast and crystalline liver catalases was quite temperature-sensitive, since it was characterized by activation energies of 16 and 10 kcal. respectively. However, unlike alteration, there was little or no effect of heat when applied to the enzyme after the irradiation. Although in the earlier experiments of Setlow on inactivation of dry catalase by deuterons (22) an increase in inactivation cross-section (*i.e.*, in probability of inactivation by a single hit) was caused by pre- and postirradiation heating, no such effect was observed in his later work on UV photoinactivation of this enzyme (23). Apparently the photoinactivation of the dry enzyme was a less temperature-sensitive process than that of the wet enzyme, as noted in Table VI, part B. A small postirradiation heat effect in the inactivation of phage by x-rays was noted by Adams and Pollard (24), but the increase in the inactivation cross-section was about ten times greater when heat was applied during the irradiation. Similar results were reported by Wood (25).

The similarity in kinetics and heat-sensitivity of photoinactivation of

intracellular catalase and of the crystalline enzyme in solution indicates that the latter was an adequate model of the former in this respect. Photoinactivation is the only phenomenon so far studied of which this is true. An aqueous solution of the crystalline enzyme–RNA complex is a satisfactory model of the intracellular protection effect, while the crystalline catalase adsorbed at an oil/water interface stabilized by cephalin or other agents is a fair model of certain aspects of the state of the intracellular enzyme (low activity, high μ_1, etc.) (26). Yet we have quite a way to go before achieving an *in vitro* model in which the state of the enzyme will be the same in all respects as that within the cell.

D. Prospect.—The next paper in this series is a study of the induced biosynthesis of yeast catalase and will show the disappearance of the Euler effect in the "unadapted" yeast cells; our data favor the hypothesis that the interface at which catalase is adsorbed within the resting cells is the surface at which it is synthesized.

SUMMARY

The effect of prolonged UV irradiation (mostly 2537 A) on the catalase activity of an aqueous yeast suspension was divisible into 4 periods. *First*, the period during which the cells lost their ability to form colonies, but during which no change in catalase activity was noted. *Second*, the period during which a considerable rise in catalase activity (Euler effect) occurred. The Euler effect was accompanied by enzyme alteration as shown by the simultaneous decrease in the activation energy of the enzyme-substrate system. However, during the initial phase of this period, as the catalase activity of the suspension began to increase, the activation energy rose to a transient level higher even than that characterizing the unaltered enzyme. Heat accelerated the rate of alteration when applied either during or after the irradiation; the activation energy for the over-all alteration reaction was 24 kcal., a value close to that recorded previously for alteration induced by chemical agents. Nevertheless, the rate-limiting step appeared to be different in the two cases. A model of these events was presented in which the primary photochemical action was on the site at which catalase is located within the cell. *Third*, a rather long period during which irradiation led to no diminution in the catalase activity of the maximally active suspension. This protection effect was duplicated *in vitro* by a model crystalline catalase–RNA system, or by adding either ribonuclease digestion products of RNA or adenine to a catalase solution prior to irradiation. Evidence was adduced that the protection effect was not a simple screening, but involved some sort of interaction between the enzyme and the nitrogenous components of RNA, an interaction which must likewise occur within the cell. Alteration induced by $CHCl_3$ did not eliminate the protection effect, but that by butanol did. The onset of photoinactivation

was due to modification of protein structure, not of RNA. *Fourth*, the period of photoinactivation of the intracellular enzyme, which was quite similar to that of the crystalline enzyme *in vitro*.

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STUDIES OF FLUID EXCHANGES BETWEEN RAT LIVER SLICES AND SIMPLE MEDIA

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(Received for publication, May 25, 1956)

When slices of mammalian tissue are incubated under conditions which depress metabolism they take up fluid from Ringer's and hypertonic solutions which contain up to twice the solute concentration of extracellular fluid (1–4). When the metabolic activity of the slices is restored towards normal the swollen tissues lose fluid and return to their original volume (4–13). These findings have been cited as indicating the inadequacy of the classical theory of osmotic equilibrium between cells and their immediate environment and have been offered as evidence that the intracellular osmotic activity is higher than, and may be twice, that of the extracellular fluid (1–5, 15–18). Presumably this osmotic gradient is established across normal living cell membranes by a process of water expulsion which steadily counteracts inward diffusion and is maintained at the expense of the resting oxidative metabolism of the cell through the mediation of phosphate bond energy (5, 6).

However, the inverse correlation of tissue swelling and metabolic activity may also be the result of a reversible shift of both solute and solvent. The present paper reports on such a transfer of both solute and water between liver slices and simple media.

Methods

The animals used in these experiments were adult male and female rats weighing 150 to 250 gm. They had been maintained on a standard laboratory diet with free access to water. The rats were killed by bleeding and their livers removed immediately. Slices of liver were cut by hand to a thickness of approximately 0.5 mm., gently blotted with cotton gauze, weighed, and then immersed in 25 to 50 ml. of various solutions at temperatures of 4, 20, and 37°C. for periods of 1 to 60 minutes. Upon removal from the solutions the slices were blotted again, reweighed, and their sodium and potassium contents determined by flame photometry. The sodium and potassium contents of unimmersed slices were determined in parallel procedures.

In separate experiments the water content of the tissues was measured as the weight loss after drying to constant weight at 105 ± 5°C. In some of these experi-

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ments the tissue slices were dried in siliconed pyrex weighing bottles, digested in nitric acid and prepared for sodium and potassium analysis. In the remaining experiments, the chloride content of the tissues was determined in slices dried in platinum crucibles, alkalinized with 1 ml. of 0.1 M $NaHCO_3$, dried again, and then ashed at 500°C. The residue was dissolved in 1 ml. of 0.4 N H_2SO_4, transferred quantitatively to small beakers with redistilled water, and analyzed for chloride content by differential potentiometric titration (21).

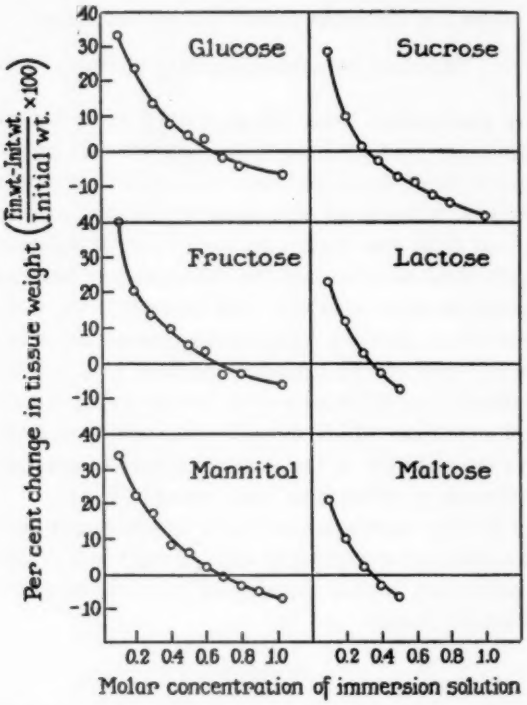

FIG. 1. Changes in the weight of liver slices immersed for 10 minutes at 20°C. in solutions of monosaccharides, and disaccharides varying in concentration from 0.1 to 1.0 molar.

RESULTS

Immersion of Fresh Liver Slices in Simple Non-Electrolyte Media.—Slices of liver immersed for 10 minutes at 20°C. in media containing glucose, fructose, or mannitol appeared to be isotonic with 0.62 to 0.68 molar solutions, since the slices gained weight in solutions which were less concentrated and lost weight in those which were more concentrated (Fig. 1, Table I). When the temperature of the immersion fluid was 37°C., the point of apparent isotonicity (P.A.I.) of the tissue did not differ significantly; but at 4°C., the P.A.I. dropped to 0.45 molar (Fig. 2). When the period of immersion was extended from 10 to 60 minutes all slices swelled significantly more at 37°

than at 20°C. although the curves were qualitatively similar at the two temperatures (Fig. 3).

When liver slices were immersed for 10 minutes in disaccharide solutions (sucrose, factose, or maltose) the P.A.I. ranged from 0.35 to 0.37 M at 20°C. and remained unchanged at temperatures of 4° and 37°C. (Figs. 1 and 4; Table I).

TABLE I

Apparent Isotonicity of Liver Slices Immersed for 10 Minutes at 4, 20, and 37°C. in Solutions of Monosaccharides, Disaccharides, and Sodium Chloride

Immersion fluid solute	Temperature	No. of experiments	Molar concentration of the apparently isoosmotic solution	
			Mean	Range
	°C.			
Glucose	4	5	0.45	0.42–0.48
	20	5	0.68	0.61–0.80
	37	5	0.64	0.60–0.68
Fructose	20	2	0.62	0.61–0.63
Mannitol	20	5	0.64	0.60–0.75
Sucrose	4	3	0.35	0.34–0.37
	20	5	0.35	0.33–0.40
	37	3	0.37	0.34–0.38
Lactose	20	4	0.36	0.31–0.36
Maltose	20	4	0.37	0.32–0.40
Sodium chloride	4	5	0.34	0.29–0.38
	20	5	0.37	0.34–0.40
	37	7	0.27	0.21–0.34

Immersion of Fresh Liver Slices in Solutions of Sodium Chloride.—When slices of liver were immersed for 10 minutes at 20°C. in 0.1 to 1.0 molar solutions of sodium chloride, fluid entered the tissues from the solutions that were less concentrated and left the tissues when the solutions were more concentrated than 0.37 (±0.02) molar. At 4°C., this point of apparent isotonicity was 0.34 (±0.04) molar; and not significantly different from the P.A.I. at 20°C. At 37°C.,[1] the P.A.I. averaged 0.27 (±0.04) molar and proved to be significantly ($p < 0.01$) lower than at 4° and 20°C. (Fig. 5).

[1] It is noteworthy that the slope of the curve relating change in tissue weight to the sodium chloride concentration of the medium was greater at 37 and 20°C. than at 4°C. rendering the measurement of the P.A.I. more sensitive at the higher temperatures.

Because of its apparent isotonicity with liver tissue, the 0.3 molar sodium chloride solution was used to study the effects of temperature and of a longer period of immersion on the shifts of water, sodium, potassium, and

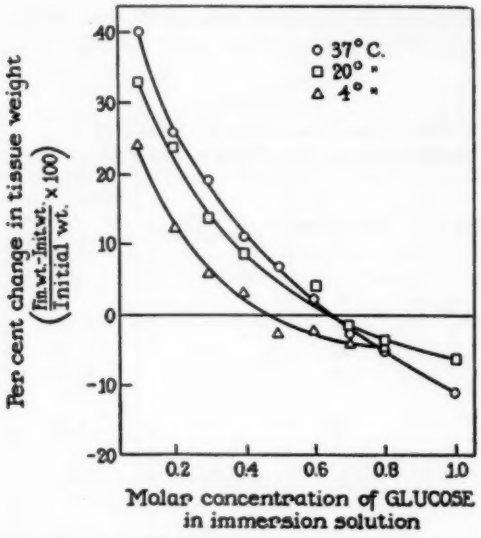

FIG. 2. Changes in the weight of liver slices immersed for 10 minutes at 4, 20, and 37°C. in solutions of glucose varying in concentration from 0.1 to 1.0 molar.

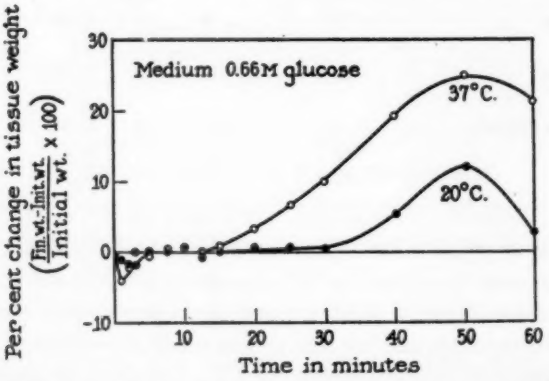

FIG. 3. Changes with time in the weight of liver slices immersed at 20 and 37°C. in 0.66 molar glucose solutions.

chloride between the slice and the bath. Typical results of this study are given in Fig. 6 and Table II. At 20°C. the slices first lost fluid for a period of 2 to 4 minutes, but after 10 to 15 minutes, began to swell. In contrast, at

37°C., the initial loss of fluid was maintained throughout the entire 60 minutes of the experiment. At all times after immersion the content of sodium and chloride was less at 37° than at 20°C., while the retention of potassium

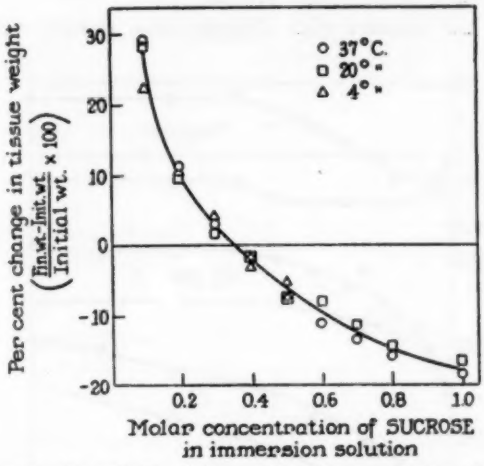

FIG. 4. Changes in the weight of liver slices immersed for 10 minutes at 4, 20, and 37°C. in solutions of sucrose varying in concentration from 0.1 to 1.0 molar.

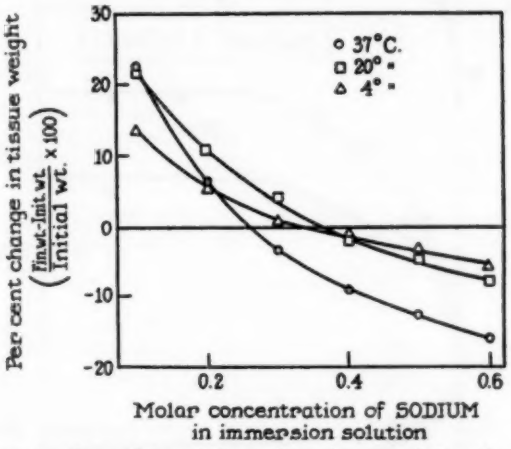

FIG. 5. Changes in the weight of liver slices immersed for 10 minutes at 4, 20, and 37°C. in solutions of sodium chloride varying in concentration from 0.05 to 0.6 molar.

was always greater at 37° than at 20°C. Thus the accumulation by the tissue of both salt and water was greater at 20° than at 37°C.

After 30 minutes of incubation at both 20 and 37°C., the sodium, potas-

sium, and water content of the slices remained constant. The chloride content of the slices was lower than the Na+K content but continued to increase for as long as 60 minutes suggesting that chloride from the medium was exchanging with other intracellular anions.

In addition to the transfer with chloride ions, sodium entered the tissue

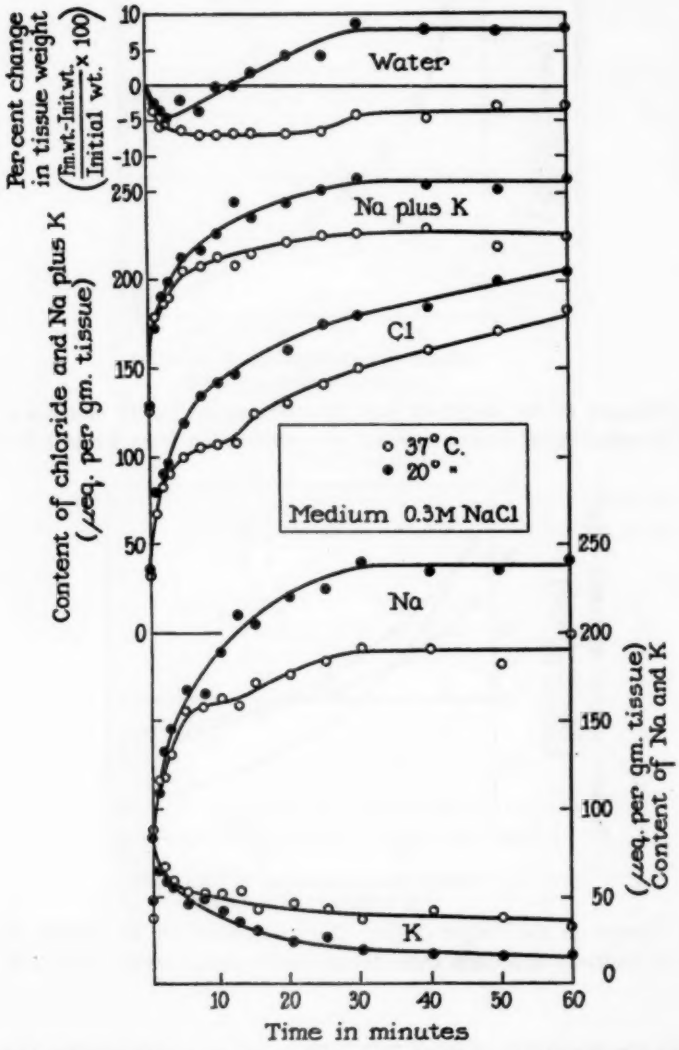
Percent change in tissue weight (Fin.wt.-Init.wt./Initial wt. x 100)
Content of chloride and Na plus K (μeq. per gm. tissue)
Water
Na plus K
Cl
o 37° C.
• 20° "
Medium 0.3M NaCl
Na
K
(μeq. per gm. tissue) Content of Na and K
Time in minutes

FIG. 6. Changes with time in the weight and the content of sodium, potassium and chloride (microequivalents per gram wet weight) of liver slices immersed at 20 and 37°C. in 0.3 molar sodium chloride solutions.

TABLE II

Difference in the Hydration and Na + K Content of Liver Slices Immersed at 20 and 37°C. in 0.3 Molar Sodium Chloride

	Incubation temperature	Control slices (unimmersed)	Duration of immersion period, *min.*			
			15	30	45	60
	°C.					
A. Per cent of total tissue mass remaining after immersion (observed)		100				
	20		95.6	94.7	95.6	94.9
	37		87.4	84.3	87.4	90.1
B. Per cent of dry substance before and after immersion (observed)		30.2				
	20		27.5	26.8	25.9	25.0
	37		28.6	28.3	27.4	25.7
C. Gm. of dry substance remaining after immersion of 1 gm. of original tissue ($10^{-4} \cdot A \cdot B$)	20		0.263	0.257	0.248	0.237
	37		0.250	0.239	0.239	0.232
D. Gm. of dry substance lost on immersion of 1 gm. of original tissue (0.302 − C)	20		0.039	0.045	0.054	0.065
	37		0.052	0.063	0.063	0.070
E. Per cent loss of dry substance $\left(\frac{D}{0.302} \cdot 100\right)$	20		12.9	14.9	17.8	21.5
	37		17.2	20.8	20.8	23.2
F. μeq. of Na per gm. wet weight of tissue before and after immersion (observed)		55				
	20		190	217	231	226
	37		166	191	201	218
G. μeq. of K per gm. wet weight of tissue before and after immersion (observed)		87				
	20		41	30	26	25
	37		54	46	41	29
H. μeq. of Na + K per gm. wet weight of tissue before and after immersion (F + G)		143				
	20		231	247	256	251
	37		221	235	243	247
I. μeq. of Na + K remaining after immersing 1 gm. of original tissue ($10^{-3} \cdot A \cdot H$)	20		221	234	245	238
	37		193	198	212	223
J. Increase of Na + K content (μeq.) on immersion of 1 gm. of original tissue (I − 143)	20		78	91	102	95
	37		50	55	69	80

TABLE II—*Concluded*

	Incubation temperature	Control slices (unimmersed)	Duration of immersion period, min.			
			15	30	45	60
	°C.					
K. Per cent tissue water before and after immersion (100 − B)		69.8				
	20		72.5	73.2	74.1	75.0
	37		71.4	71.7	72.6	74.3
L. Tissue water content after immersion expressed as gm. H_2O per gm. of original tissue. ($10^{-4} \cdot A \cdot K$)	20		0.693	0.692	0.709	0.712
	37		0.624	0.604	0.634	0.670
M. Gm. of water gained or lost from 1 gm. of original tissue after immersion. (0.698 − L)	20		−0.005	−0.006	+0.011	+0.014
	37		−0.074	−0.094	−0.064	−0.028
N. μeq. of Na + K gained by 1 gm. original tissue as a result of temperature change from 37 to 20°C. ($J_{20°C.} - J_{37°C.}$)	20–37		28	36	33	15
O. Gm. of water gained by 1 gm. of original tissue as a result of temperature change from 37 to 20°C. ($M_{20°C.} - M_{37°C.}$)	20–37		0.069	0.088	0.075	0.042
P. μeq. of Na + K per gm. of water gained by the tissue from the medium as a result of temperature change from 37 to 20°C. (N/O)	20–37		405	409	440	358

Each value represents the average of a group of five experiments.

slice by exchanging with potassium. A comparison of the analyses obtained on slices immersed at 20 and at 37°C. showed that the change in the concentration of sodium plus potassium corresponded approximately to the change in the concentration of chloride (Fig. 6); this change averaged 344 (±101) μeq. per gm. change in tissue water. Thus, with change in temperature from 37 to 20°C. or from 20 to 37°C., the slice may be considered to gain or lose 688 (±202) m.osM per kilogram of total shifting fluid. If the immersion fluid, which contains 600 m.osM per liter (activity coefficient taken as 1), be regarded as entering and leaving the slices in bulk, other mechanisms are necessary to provide additional osmotically active intracellular solute when the observed concentration of the shifting fluid is less than 600

m.osM per liter, and to inactivate such intracellular solute when the shifting fluid has a higher osmolar concentration.

DISCUSSION

Deyrup has shown that slices of rat kidney swell in physiological fluids or in equally concentrated solutions of monosaccharides, but not in 0.3 molar solutions of disaccharides (14). The results of the present study with liver slices confirm Deyrup's observations on renal tissue, and the differential effects of temperature are in accord with her suggestion that the swelling is due to ingress of water accompanying the primary entry of solute into the tissue cells. Presumably the greater swelling of liver slices in glucose solutions at 37 and 20°C. as compared with slices immersed at 4°C. is due to the more efficient inward transport, accumulation and/or metabolism of the immersion fluid solute (glucose) at the higher temperatures.

One of us has reported previously (22) on the change of the sodium content of liver slices immersed for varying periods in solutions of varying sodium concentration. When the sodium concentration of the medium, M_{Na} was held constant and the time of immersion varied, it was found that the slice gained sodium till the 8th minute, and thereafter its sodium content remained constant for 10 to 15 minutes; when M_{Na} was varied and the slice immersed for 10 minutes, the entry of sodium into the tissue was found to be proportional to M_{Na} and independent of the degree and direction of the fluid movement as judged by the change in the weight of the slice. At the time we interpreted these findings to mean that the medium had come into equilibrium with the rapidly exchanging (extracellular) phases of the slice within the first 10 minutes after immersion, but that the solute of the medium had not yet penetrated the less accessible (intracellular) phases.

This interpretation implied that the solute concentration of the intracellular phases of the slice was 0.6 to 0.8 osmoles per liter, that is, the osmolar concentration of the sodium chloride solution in which the slice neither gained nor lost weight. However, this interpretation of the earlier experiments did not account for the subsequent finding that the weight of the tissue slices immersed in hypertonic solutions first decreased before reversing to regain or exceed the original value (Figs. 3 and 6). Also the comparison of electrolyte content and hydration of liver slices at 20 and 37°C. suggested that the greater swelling at the lower temperature was due to the depressed metabolic activity of the cells with consequent failure of solute (sodium and chloride) extrusion. These latter findings do not support the assumption that liver cells respond to changes in external osmotic pressure by movement of water alone but rather support the conclusion of Mudge (12) and Leaf (23) that cells adjust to osmotic changes with electrolyte shifts and do not react as simple osmometers.

In general, it was found that there was sufficient Na+K entering or leaving the slices to account for the observed changes in tissue volume in terms of movement of medium in bulk. In some experiments the amount of Na+K accumulated in the tissue when the temperature was lowered exceeded that required for simple bulk transfer of medium. For example, in the group of experiments presented in Table II an average of 400 μeq. of Na+K accompanied each gram of water entering the slice when the temperature of incubation was lowered from 37 to 20°C. It is possible that under the conditions of these experiments there occurred a net aggregation of intracellular constituents, decreasing the intracellular osmotic activity concomitantly with or shortly after the failure of sodium chloride extrusion.

In other experiments it was found that only 250 μeq of Na+K accompanied each gram of water entering or leaving the slices. In these experiments the movement of medium in bulk would explain only five-sixths of the tissue swelling observed when the incubation temperature is lowered from 37 to 20°C.; the residual swelling may be accounted for by movement of water alone in response to a gradient of 100 m.osm per liter developed across cell and/or subcellular membranes. This magnitude of intracellular hypertonicity is in accord with the cryoscopic measurements of Appelboom, Brodsky, Dennis, Rehm, Miley, and Diamond (24) and the rapid (19) and presumably reversible (20) breakdown of ATP, hexose esters, creatine phosphate, and glycogen *in vitro*.

Inasmuch as relocation of solute depends on metabolic activity, it is difficult to consider disaggregation or breakdown of intracellular constituents and solute transport processes as independent factors influencing the water exchange between cells and extracellular fluid or between subcellular structures and cytoplasm; an adequate theoretical treatment of this problem would require detailed knowledge of the mechanisms of the major relevant solute fluxes and the precise nature, sequence, and relationship of the coupled chemical reactions. As such knowledge accumulates it may become possible to distinguish between the movement of preexisting particles and the appearance of new particles and to assess quantitatively the role of each as factors in cell hydration. For the present, however, it seems more important to develop direct methods of study, such as improved techniques for measuring the colligative properties of tissue preparations (24), which will permit a valid interpretation of the findings now offered as evidence that the free energy of water is lower within cells than in extracellular fluid.

SUMMARY

1. The exchange of fluid between slices of rat liver and solutions of monosaccharides, disaccharides, and sodium chloride has been studied in relation to the temperature of incubation. The point of apparent isotonicity (P.A.I.)

of the tissue was defined as the concentration of the solution in which the slices neither gained nor lost weight after immersion for a period of 10 minutes.

2. In solutions of glucose, the P.A.I. of the slices was significantly lower at 4°C. than at 20°C. but similar at 20°C. and 37°C. Upon immersion for 15 to 60 minutes in 0.66 molar glucose the slices always swelled more at 37°C. than at 20°C. In solutions of sucrose change in the temperature of incubation was without effect on the hydration of the tissues.

3. In solutions of sodium chloride, the P.A.I. and the content of water, chloride, and sodium plus potassium were lower at 37°C. than at 20°C.

4. These findings emphasize the role of translocation of solute in providing an osmotic gradient for the movement of water between the tissue slices and the media.

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THE FREEZING POINT DEPRESSION OF MAMMALIAN TISSUES IN RELATION TO THE QUESTION OF OSMOTIC ACTIVITY OF CELL FLUID*

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(Received for publication, March 16, 1956)

INTRODUCTION

The classic concept that intracellular fluid is isoosmotic with the extracellular fluid has never been rigorously established.

Evidence in favor of isoosmotic cell fluid has been adduced from the following: (*a*) The freezing point depression and vapor tension lowering of erythrocytes have been shown to be equal to those of plasma (1–6). Moreover, erythrocytes behave as nearly perfect osmometers when suspended in solutions of varying osmolarity (7); (*b*) the estimated sum of the molal concentrations of solutes in erythrocytes and muscle (8–11); (*c*) changes in serum osmolarity after injection of hypertonic solutions into intact animals (12–14).

On the other hand, evidence casting doubt on the concept of isoosmotic cell fluid has been accumulating for a long time. Sabbatani found that the freezing point depression of many dog tissues (removed 2 to 60 minutes after death) was greater than that of blood (15). His results were confirmed by Collip (16) who explained the high freezing point depression of excised tissue by assuming the activation of osmotically inert particles in the tissue after removal. Along other lines, the observation of tissue swelling in isotonic solutions has been made repeatedly by many early workers (17–20). Swelling was usually accounted for by the implicit assumption of cellular isotonicity initially with the subsequent production of osmotically active particles derived from the catabolic reactions of glycogen or proteins.

Recent data on swelling of tissue slices in isotonic solutions (21–31) and on melting point measurements of frozen tissues (32, 33) have posed a new challenge

*This investigation was supported in part by research grants A-461-C4 and RG-3503(C3) from the National Institutes of Health, Public Health Service, and in part by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, contracts DA-49-007-MD-481 and DA-49-007-MD-279, and in part by a grant from the Kentucky Medical Research Commission.

‡ Established Investigator of the American Heart Association.

to the validity of the classic concept. Thus, freshly excised slices of mammalian tissue swell in isotonic, but not in hypertonic solutions, and the swelling in isotonic media could be reversed by oxygenation of the ambient fluid. These results led to the suggestion that the cell fluid *in vivo* is hypertonic to extracellular fluid, and that the osmotic gradient is normally maintained by continuously pumping water out of the cell (21, 22). However, Deyrup (29) found that tissues do not swell in isotonic sucrose, but do swell in isotonic sodium chloride, glucose, and Krebs-Ringer, and after oxygenation, return to normal volume in Krebs-Ringer or in isotonic sodium chloride solutions. She suggested that water pumps alone could not explain these phenomena, and that shrinking was apparently dependent upon the presence of ions in the ambient solution.

Ionic shifts occurring concomitantly with swelling or with oxygenation of tissue slices have been reported (34–39). Schwartz (30) placed tissue slices in a series of sodium chloride solutions and found that the sodium space of the slices after 10 minutes of immersion amounted to values close enough to those of extracellular fluid to suggest that the effect of immersion within the first 10 to 15 minutes was to equilibrate the interstitial fluid with the solutions. Subsequently, in studying temperature effects on the swelling of tissue slices immersed in solutions of sodium chloride, monosaccharides, and disaccharides, Itoh and Schwartz (31) were able to account for the observed volume changes of the tissues in terms of solute shifts between the slices and the ambient media.

Such data on swelling and composition changes of immersed tissues stimulated some to make direct measurements of the colligative properties of tissue juices obtained under more elegant conditions than those employed by earlier workers. Although all the recent cryoscopic data showed that the freezing point depression of freshly excised, frozen tissues was greater than that of plasma (32–33, 40–44), diametrically opposing conclusions were drawn by different investigators. Thus, Conway and McCormack (42) inferred that cell fluid is isotonic because the calculated tissue osmolarity of saline-dilute homogenates, extrapolated to zero time, approximated the plasma osmolarity. Similarly, Howard (40) claimed that cell fluid is isotonic because the freezing point of normal muscle and testicular tissue in rats was the same as that of plasma. However, she, like others, noted that the freezing point depression of liver was higher than that of plasma. On the other hand, Opie inferred that cell fluids must be hypertonic to plasma, since the melting point of frozen tissue powder was lower than that of plasma (32). Wirz *et al.* drew similar inferences on the tonicity of renal medullary cells on the basis of microscopic visualization of melting in the frozen tissue at a temperature less than that of plasma melting point (33).

None of the aforementioned experiments contained data pertinent to the effect of the technique of preparation on the structural or physical properties of the tissues. While tissue swelling experiments were performed on slices kept at room temperature or at 38°C., cryoscopic experiments were usually per-

formed on pulverized powder of tissues in liquid N_2 or O_2 kept at about 0°C. during the actual measurements. In short, the experimental conditions that differed among all reported data included the degree of disruption of tissue architecture in the sample used, the individual ion flux of slices, the time interval between tissue excision and determination, the effect of temperature during the actual measurement, and the possible effects of various diluent solutions.

The primary purpose of the present report was to study the cryoscopic behavior of tissue homogenates prepared in different ways. To obtain samples containing intracellular fluid, methods were devised to insure cell destruction in the homogenates. What follows will concern: (*a*) an attempt to establish the validity of the cryoscopic method as a measure of the osmotic activity of tissue homogenates; (*b*) an attempt to determine whether or not cells are destroyed in tissue homogenates prepared in different ways; and (*c*) the effect of various diluents on the calculated freezing point depression of different tissue homogenates.

Methods

Tissues, removed without hemostasis from dogs under the influence of nembutal or amytal, were immersed immediately (within 5 to 10 seconds or less) in liquid nitrogen or in isopentane cooled with liquid N_2. The frozen blocks of tissues were treated in one of two ways: (1) Some blocks were crushed in the Carver hydraulic press, and the tissue juice so obtained was placed immediately in test tubes for determination of freezing point depression. At first, the crushing was done with the press kept at room temperature, but the temperature of homogenates oozing out of the press reached +13° within 2 to 3 minutes. Therefore, crushing was performed with the press cooled to +2–3°C., so that the temperature of the homogenates obtained rarely exceeded +1–2°C. before they were placed in the osmometer. (2) Other blocks of frozen tissue removed from liquid nitrogen were placed in a mortar, covered with liquid N_2, and pulverized to a fine powder. Approximately 1.5 to 2.0 gm. of the frozen powder were quickly scraped into the appropriate tube for determination of freezing point depression. When aliquots of the same tissue of one dog were processed at the same time through "warm" and cool presses and by means of the mortar and pestle, the freezing point data from all three procedures showed no significant differences.

At first, all measurements of osmotic activity were made on homogenates without any added diluent solution. Later, when testing the cryoscopic method, and when testing the time course of osmotic activity in homogenates, various diluent solutions were added. Determinations of freezing point depression of tissue samples were performed in a Fiske osmometer using a thermistor as the temperature-sensing element.

RESULTS

Preliminary Tests

In earlier experiments, freezing point determinations were made on serum, plasma, red cells, whole blood, liver, kidney, pancreas, stomach, lung, muscle,

brain, and spleen. The data indicated that erythrocytes were isotonic to plasma or serum, while homogenates of all other tissue (except about one-half of the spleen samples) were hypertonic to plasma. Some of the high values (827 for liver, 538 for kidney) are from determinations on tissues which had remained at room temperature or at 0°C. for periods as long as several hours.

A superficial examination of the data in Table I might lead one to suspect the existence of a hypertonic tissue fluid. However, practical objections to the experimental conditions became apparent. (1) There was a wide scatter of data

TABLE I

Values of Osmotic Activity Determined Cryoscopically on Serum, Plasma, Erythrocytes, Whole Blood, and Various Tissues

Tissue		Osmolarity	
		Average	Range
Serum	(7)	286	266–300
Plasma	(32)	300	280–325
Red blood cells—clotted	(8)	310	285–392
Red blood cells—heparinized	(13)	325	266–342
Whole blood	(6)	289	277–300
Liver	(24)	413	314–827
Kidney	(23)	434	337–538
Spleen	(11)	312	286–399
Muscle	(9)	359	297–390
Pancreas	(5)	355	335–390
Gastric mucosa	(3)	328	315–340
Brain	(2)	313	306–319
Intestine	(1)	422	

Numbers in parentheses designate the number of experiments performed on each tissue listed. The value for each experiment was the average of several freezing point determinations on each tissue sample.

indicating poor reproducibility of freezing point values in tissues of different dogs. Occasionally, even the juice of a single organ would yield divergent data. On the other hand, standard salt solutions, or blood from a single dog would yield reproducible freezing point values. (2) The time required to reach the freezing point was longer for tissue juice than for plasma, red blood cells, or salt solutions. Perhaps the heat transfers between solid and liquid phases and environment at the freezing point were changed in a gel or protein system like tissue juice. Thus, the viscosity, high protein content, or gel state form of solutions in homogenates might have caused the observed freezing point depression to be spuriously high. Such objections led to considerable skepticism as to the validity of cryoscopic measurements of undiluted tissue homogenates. There-

fore, experiments were designed to check the validity of the freezing point technique under present experimental conditions. If tissue fluids were spuriously hypertonic because of the high protein content or viscosity of cell fluid, then dilution of the homogenate with water would be an obvious way to reduce these factors.

Testing the Validity of the Cryoscopic Technique Applied to Tissue Homogenates

Dilution tests were done as follows: Tissue juice from 100 gm. of kidney or liver processed in the Carver press was homogenized in a Waring blendor for 3 to 5 minutes. Weighed aliquots of the homogenate plus weighed aliquots of distilled water were placed in flasks immersed in an ice bath. Portions of the

TABLE II

Schematic Table Showing the Experimental Design Used in Dilution Tests

Tube No.	Tissue	Tissue water	Added water	Dilution factor
	gm.	gm.	gm.	
1	10	7.5	0	1
2	10	7.5	1.1	1.06
3	10	7.5	2.5	1.33
4	10	7.5	4.5	1.60
5	10	7.5	7.5	2
6	10	7.5	15.0	3
7	10	7.5	22.5	4
8	10	7.5	52.5	8

cooled liver or kidney homogenates were transferred to test tubes for cryoscopic measurements. Table II presents an hypothetical protocol for the scheme of serial dilution used. The chronologic order of determinations at different dilution levels was randomized. The interval between pressing the frozen block of tissue and the freezing point determination was approximately 30 minutes in most cases. When a single aliquot of tissue was subjected to the aforementioned treatment, it was found that osmolarity was 450 to 500 m.osM/liter and would show but small change after immersion in an ice bath for the next 30 to 60 minutes.

If the apparent freezing point depression of the undiluted homogenate were erroneously high due to factors interfering with heat exchanges in the freezing mixture, the progressive dilution of the homogenates should reduce the calculated values of osmotic activity so that they would approach those of plasma. Fig. 1 illustrates calculated values of tissue osmolarity *versus* dilution in a representative experiment. A total of 9 experiments was performed, and it was found that the calculated osmolarity did not change significantly over an eightfold dilution range. Such data conform with those expected on the assumption that

freezing point measurements of undiluted homogenates are accurate. The calculations did not correct for changing activity coefficients of the tissue electrolytes at varying degrees of dilution. But even had this correction been applied, the osmolarity calculated from the most dilute sample would have been but a few per cent lower than the value shown, and consequently, still over 1½ times the value of plasma osmolarity.

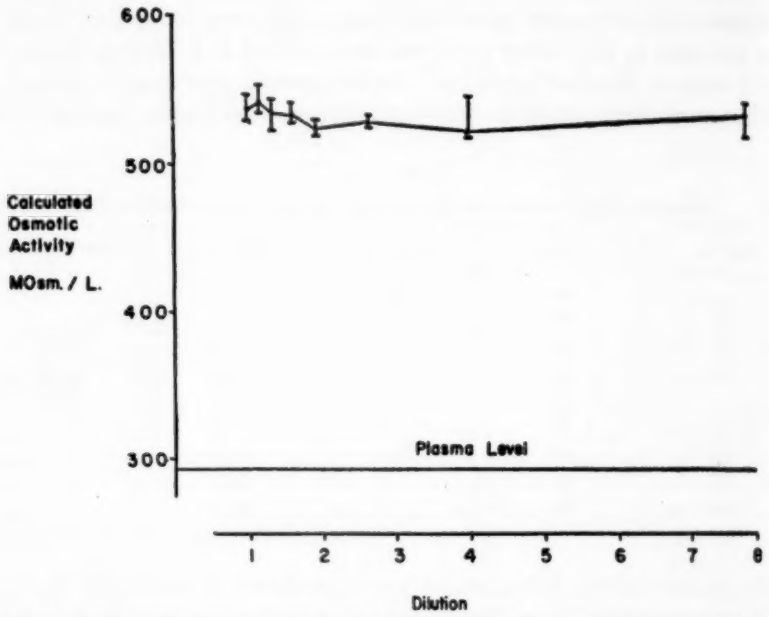

FIG. 1. Calculated osmotic activity of tissue *vs.* dilution. Each point plotted represents the average of 5 determinations of freezing point depression. The range of each set of values is indicated by the length of the vertical line.

The foregoing experiments indicate that the osmolarity of undiluted homogenates is apparently higher than that of plasma. This does not necessarily mean that the fluid within a living cell *in situ* is hypertonic to the surrounding extracellular fluid.

The Effect of Time and Temperature

Since time of standing and environmental temperature have been found to influence the osmotic activity of undiluted homogenates (41–44), a systematic investigation of the effect of such factors was undertaken.

Samples of liver homogenate were prepared in the prescribed manner as quickly as possible, and the first determination of freezing point depression was obtained within 5 minutes after removing the homogenate from the cooled press or from the mortar and pestle. The undiluted homogenates were kept in

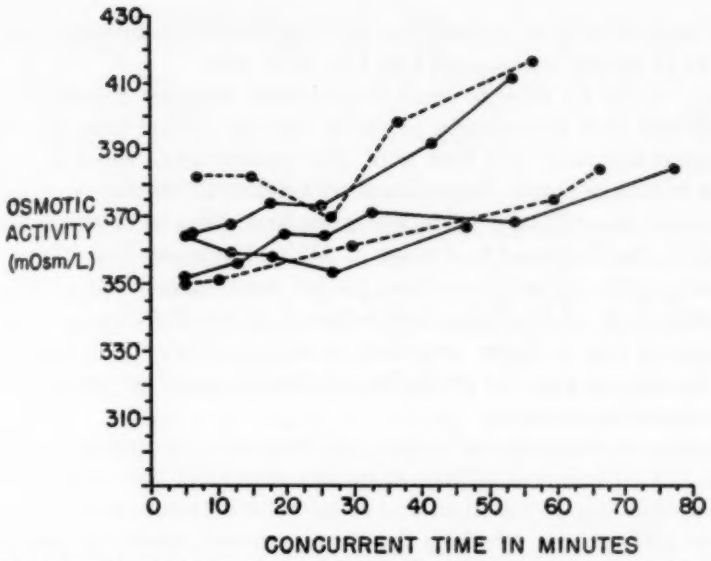

FIG. 2 *a*. Osmotic activity *vs.* time. Undiluted liver homogenate prepared in Carver press. Zero time refers to the instant pressure was applied to the blocks of frozen tissue in the press. Plasma osmotic activity = 287 to 314 m.osM/liter.

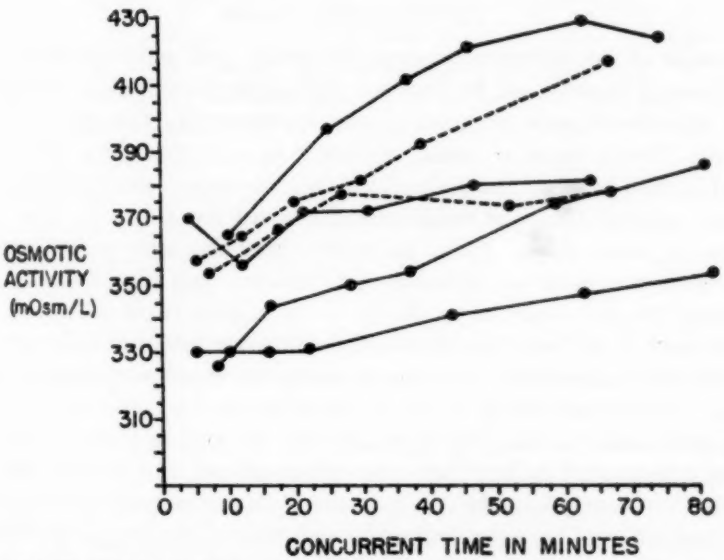

FIG. 2 *b*. Osmotic activity *vs.* time. Undiluted, pulverized liver powder prepared in the mortar under liquid N_2. Zero time refers to the time the powder, previously covered by liquid N_2, reached a temperature of 0°C. The time interval required for such thawing was no more than 1 to 2 minutes. Plasma osmotic activity = 287 to 314 m.osM/liter. Data of both Fig. 2 *a* and Fig. 2 *b* are from the same dogs.

an ice bath or at room temperature, and osmolarity measurements were made at 10 to 15 minute intervals for 1 to 2 hours or more.

Figs. 2 *a* and 2 *b* show patterns of concurrent increases of osmotic activity in undiluted liver homogenates prepared from the Carver press and with the the mortar and pestle and kept at 0°. The pattern about which data fell was similar in both instances. Inspection of both figures shows that the values after 3 to 10 minutes at 0° were 325 to 383 m.osM/liter. After 60 to 90 minutes at 0°, osmolarity had increased to a range of 340 to 430 m.osM/liter. Linear extrapolation of these curves to zero time, yielded osmolarities of 305 to 375 m.osM/liter, with 9 out of 10 extrapolated values in excess of 330 m.osM/liter. Such extrapolated values, higher than those of plasma osmolarity did not conform with the data or with the predictions of Conway based on extrapolations in saline-diluted homogenates.

The same experiments have been performed at room temperature (25°C.) and at 38°C. The rate of increase of osmotic activity of the warmed homogenates was definitely greater than that of the cooled homogenates. For example, at room temperature juice from the Carver pressed showed an osmolarity of 370 m.osM/liter in 5 minutes and over 500 m.osM/liter in 30 minutes. Results on frozen tissue powder from the mortar were closely similar to those of the Carver press data, both at 25°C. and at 38°C.

Effect of Isotonic Diluent

Because of the difference between our results with undiluted homogenates and those of Conway and McCormack with saline-diluted tissue homogenates (42), experiments were designed to test systematically the effects of saline diluents. Frozen blocks of tissue, pulverized in a mortar with a pestle under liquid N_2 (and in some cases under liquid O_2), were mixed with tared aliquots of isotonic saline at 0°C. The weight of saline added was about the same as the weight of whole tissue. Then, successive determinations of freezing point depression were made on the saline-tissue mixture (see Figs. 4 and 5). After obtaining the dry weight on an aliquot of the original batch of tissue powder, calculations of the tissue osmotic activity were made, and the results therefrom plotted graphically against time. Linear extrapolation of these calculated values to zero time yielded values of 310 to 350 mosM per liter of tissue water. The fairly wide scatter of data was similar to that reported by Conway. However, values extrapolated to zero time were about 10 per cent greater than that reported by Conway from the zero intercept of a linear regression equation (42).

Consideration of both the similarities and differences between results of the present report and those of Conway led to the following possibilities: Conway's calculations assume complete destruction of cells as well as instantaneous diffusion and mixing of intracellular with ambient fluids. However, if the cells of a tissue powder diluted in isotonic saline were intact or in clumps, and if diffusion of cell solute and water were slow, then even if the cell fluid were

hypertonic to the ambient, the initial freezing point depression of the cell-ambient mixture would approach that of the isotonic saline since it would freeze first. Even with a hypertonic cell-free homogenate diluted with isotonic saline, and in which there is incomplete mixing, the freezing point depression of the mixture would also approach that of isotonic saline.

Microscopic Examination of Tissues

Since some of the aforementioned considerations are based on the presence of intact cells, it was pertinent to examine the homogenates histologically. Smears of freshly prepared liver and kidney homogenates prepared with the mortar and pestle and from the Carver press, were fixed immediately in alcohol-ether and stained with hematoxylin and eosin. Figs. 3 *a* and 3 *b* are representative photomicrographs of liver homogenate. In the Carver press homogenate (Fig. 3 *a*) there appear many free floating nuclei and particulate bodies, but only an occasional intact cell. Several fields in each of 35 slides were examined. On the other hand, the mortar and pestle preparation (Fig. 3 *b*) had many clumps of intact cells. This tissue had been pulverized as vigorously as possible by hand for 15 to 20 minutes under liquid N_2. Only a fine powder remained in the mortar. The field shown in the figure is fairly representative of several fields examined in each of 35 such slides.

Thus, microscopic inspection of the tissues showed that the bulk of the cells had been disrupted by processing through a Carver press, while large numbers of cells and clumps of cells remained intact after pulverizing in a mortar with pestle as Conway and McCormack (42) and Opie (32) did. In view of the presence of clumps of intact cells in the mortar and pestle preparations, we questioned the validity of calculations like those of Conway which assume complete mixing of cell and ambient fluids as well as an instantaneous state of diffusion equilibrium between the two aqueous phases. Even if the cells were uniformly distributed through a perfectly homogeneous diluent solution, the rate of achievement of a diffusion equilibrium between the two phases could be delayed.

Effect of Varying Osmolarity of the Diluent

In Conway's experiments, a diluent fluid, isotonic saline, was added to frozen tissue which had been ground to a fine powder in a mortar, and the freezing point depression of the final mixture was determined repeatedly against time. The "tissue" osmotic activity was calculated from the assumption that the total number of osmotically active particles in the final mixture was equal to the total number of osmoles in the tissue and diluent. Mathematically,

$$(O_{\text{obs.}})(D_{H_2O} + T_{H_2O}) = (O_{\text{tissue}})(T_{H_2O}) + (O_{\text{dil.}})(D_{H_2O}) \quad (1)$$

or

$$O_{\text{tissue}} = (O_{\text{obs.}})\left(\frac{D_{H_2O} + T_{H_2O}}{T_{H_2O}}\right) - (O_{\text{dil.}})\left(\frac{D_{H_2O}}{T_{H_2O}}\right) \quad (2)$$

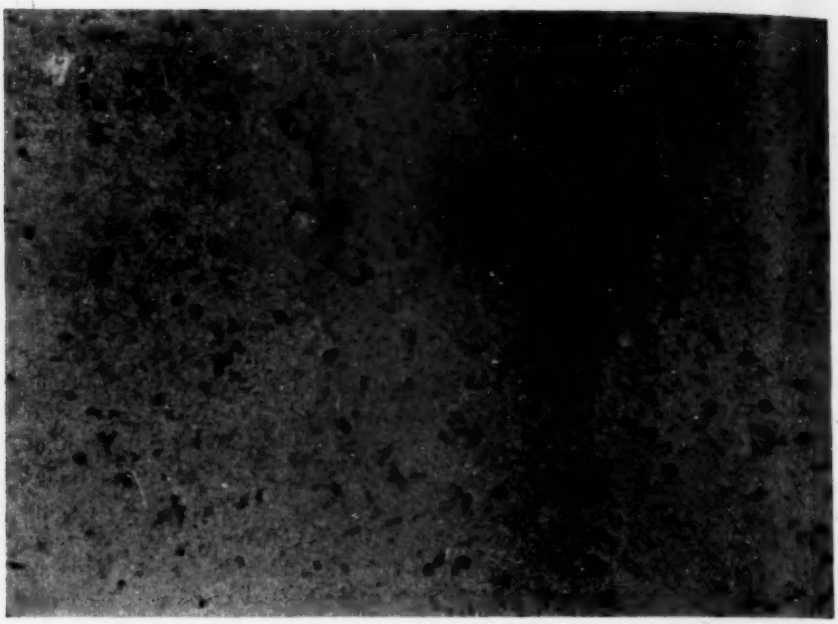

FIG. 3 *a*. Hematoxylin and eosin stain of liver juice after processing a frozen block of tissue through the Carver press.

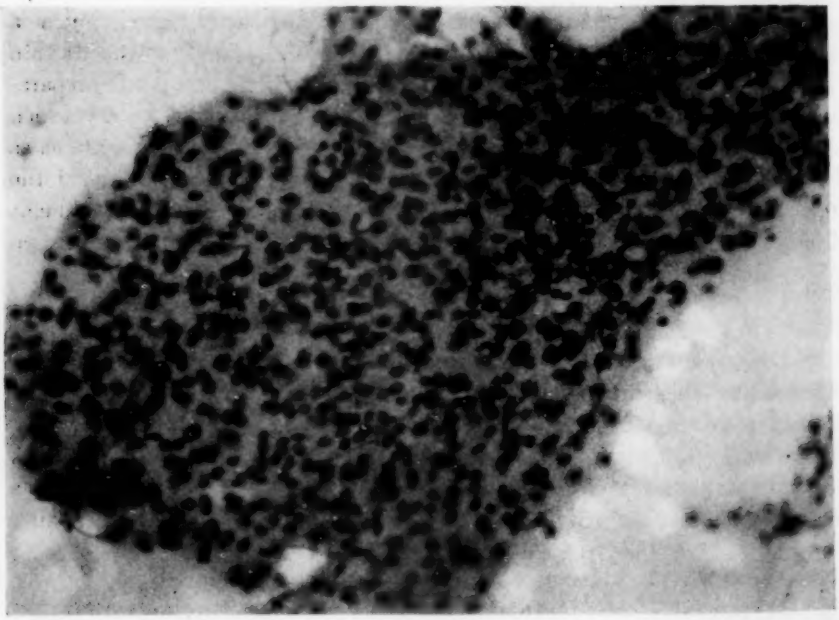

FIG. 3 *b*. Hematoxylin and eosin stain of liver powder after pulverizing frozen blocks of the tissue in a mortar.

in which O_{tissue} is the calculated tissue osmotic activity, $O_{obs.}$, the observed osmotic activity of tissue plus diluent, $O_{dil.}$, the osmotic activity of the diluent solution, D_{H_2O}, the weight of diluent solution, and T_{H_2O}, the weight of tissue water determined by dry weight measurement. The assumption implicit in this equation is that there is complete and instantaneous mixing and diffusion of all the water and osmotically active particles. If complete mixing did not occur, there would be two or more aqueous phases present. Even with com-

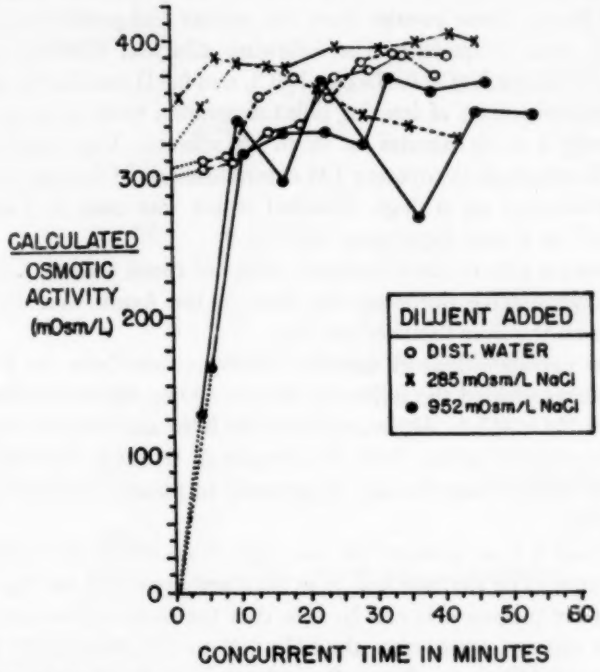

FIG. 4. Calculated osmotic activity *vs.* time. Liver powder prepared in the mortar was mixed with various diluents represented by symbols in the panel. Extrapolation shown by dotted lines.

plete mixing, an instantaneous diffusion equilibrium between the two phases would be required to satisfy the above equation. In either case, the freezing point of the final mixture would approach that of the phase of lowest osmotic activity.

If the added diluent were isotonic, and if no mixing or diffusion occurred between hypertonic cells and diluent, both the observed osmolarity of the mixture and the calculated osmolarity of the tissue would appear isotonic. In general, addition of an isotonic diluent to a hypertonic tissue phase would result in apparent isotonicity of both the mixture and the tissue, providing no equilibration occurred between the two phases.

With little or no equilibration, addition of a diluent of higher osmolarity than that of tissue could lead to a negative value for the calculated tissue osmolarity. In this case, the observed osmolarity would approach that of the aqueous tissue phase since it would freeze before a hypertonic ambient solution. Then the application of equation (2) would yield spuriously low, or even negative, values for O_{tissue} depending on the magnitude of the diluent osmolarity ($O_{dil.}$).

The following experiments were planned to determine whether or not the calculated tissue osmolarity was a function of the osmolarity of diluent. Tared aliquots of frozen tissue powder from the mortar and pestle preparation were mixed with tared aliquots of the following diluents: distilled water, 0.285 osM NaCl, 0.574 osM NaCl, 0.675 osM NaCl, and 0.931 osM NaCl. In each case, repeated determinations of freezing point depression were made on the diluted mixtures every 5 to 10 minutes for 90 to 120 minutes. Under such conditions, a total of 18 experiments covering 128 determinations of freezing point depression was performed on 6 dogs. Distilled water was used in 7 experiments, isotonic NaCl in 2, and hypertonic NaCl in 9.

Fig. 4 shows a plot of the calculated values of tissue osmotic activity *versus* time. For comparative purposes, the data in the figure were taken from 6 experiments on the liver tissue of one dog.

The linear extrapolations of osmotic activity values from the homogenate-diluent mixtures yielded the following values: 300 to 305 m.osM/liter for liver and distilled H_2O; 317 to 340 m.osM/liter for liver and isotonic saline; 0, for liver and hypertonic saline. After 20 minutes of repeated determinations, all the six liver homogenates became hypertonic to plasma osmolarity of 295 to 300 m.osM/liter.

Figs. 5 *a* and 5 *b* are plots of the raw data from which the values of Fig. 4 were calculated. The derived and raw data are presented on the same scale for comparative purposes. It can be seen that the observed osmolarities of the tissue-water mixture were approximately 120 to 125 m.osM/liter in the first 5 minutes, and 150 m.osM/liter after 50 minutes. In the tissue–hypertonic saline mixture, osmolarities were 610 to 670 in the first 5 minutes and nearly 700 m.osM/liter after 50 minutes. In both diluents, small fluctuations in observed values become exaggerated in the calculated results because of the dilution effect.

The results with hypertonic saline are compatible with the predictions of a "delayed diffusion" hypothesis when a calculation, like that in equation (1) is applied to a system of intact cells in an ambient solution. Superficially, the data from water-diluted homogenates did not conform to the results expected on the basis of incomplete or delayed diffusion processes between ambient and cell fluid. One would expect that the extrapolated value of water-diluted homogenates would be zero, in the limiting case of zero diffusion, (*i.e.* impermeable cells); or would approach zero in the case of delayed diffusion. However, it is probable that distilled water can disrupt cells more effectively

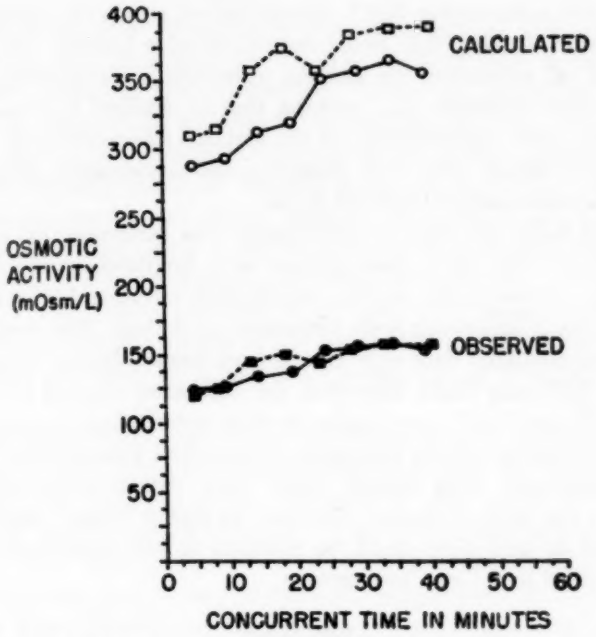

FIG. 5 *a*. Osmotic activity *vs*. concurrent time. Liver plus distilled water. Observed osmolarity of the mixture, and calculated osmolarity of the liver tissue.

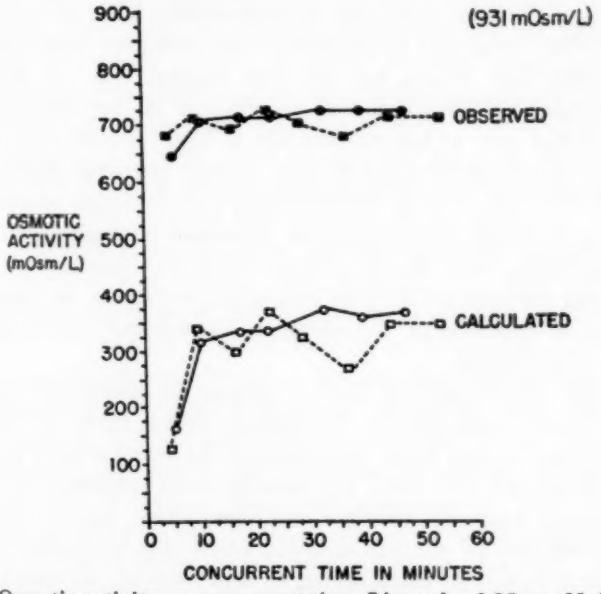

FIG. 5 *b*. Osmotic activity *vs*. concurrent time. Liver plus 0.95 OSM NaCl. Observed osmolarity of the mixture, and calculated osmolarity of liver.

than can 950 milliosmolar NaCl. Disruption of significant numbers of cells by addition of distilled H_2O would reduce diffusion barriers and favor rapid diffusion of cell solute into the ambient water which would produce an osmolarity like that observed; *i.e.*, between that of distilled H_2O and cell fluid. On the other hand, hypertonicity of the ambient solution relative to cell fluid would tend to shrink cells, favor clumping, and consequently reduce diffusion processes between ambient and cell fluids.

A similar series of dilution experiments was performed on homogenates without intact cells. Such homogenates were prepared, as described previously in the Carver press. Sixteen experiments covering 121 determinations of freezing point depression were performed on 4 dogs. The results of calculated and extrapolated data were erratic from tissues diluted in both distilled water and 0.574 osM NaCl. However, the data after dilution of the cell-free juice in 0.675 osM NaCl were similar to those noted after dilution of the cell-rich powder from the mortar and pestle preparation. This suggests that clumping of homogenate, even without intact cells, delays mixing and diffusion between the two aqueous phases. Thus the calculated "tissue" osmotic activity extrapolated to zero time could be spurious in any experiment wherein a diluent is added to a tissue homogenate.

DISCUSSION

Although the freezing point depression of undiluted homogenates is greater than that of plasma, it does not necessarily mean that cell fluid of intact tissues in the animal is hypertonic to extracellular fluid. This consideration may imply that cryoscopic measurements on undiluted tissue homogenates yield spuriously high results, but our data suggest that the measurements are at least qualitatively valid. Our experiments do not exclude the possibility that factors such as low temperature or high pressure could alter physical properties of cell proteins and thereby increase the tissue osmotic activity. If this were the case, even an instantaneous determination of freezing point depression on an undiluted homogenate (processed through liquid N_2) would give a value higher than that of plasma. Thus, the experimental conditions present during preparation of, and during measurements on tissue must be examined in all experiments purporting to demonstrate either cellular isotonicity or hypertonicity.

Conway has stated that cell fluid is isotonic to plasma. He mixed equal weights of frozen tissue powder and isotonic saline, made successive determinations of the freezing point depression of the mixture kept at 0°C., and calculated, from such raw data, the freezing point depression of the tissue. His calculations assumed complete cell destruction and/or instantaneous mixing and diffusion of cell and ambient fluids. The estimated values were plotted against time, and extrapolated to zero time by calculated regression

line applied to the data. Since the extrapolated value at zero time approximated that of plasma osmolarity, Conway deduced that tissues must be isotonic (42). Obviously such calculations ignore the possibility of delayed mixing and diffusion of solutes between cell and ambient fluids in homogenates containing intact cells. The instantaneous freezing point depression (or the extrapolated freezing point) of isotonic or hypertonic cells in an isotonic ambient solution would have to be isotonic, since the isotonic phase freezes prior to the hypertonic phase. Consequently, Conway's experiment could not have distinguished between isotonic and hypertonic cell fluid.

Howard has also claimed that tissues are isotonic to plasma (40). Her data, obtained from inserting a thermocouple between pieces of intact tissue could have provided a measure of extracellular rather than of intracellular osmotic activity.

Other workers have claimed, on the basis of cryoscopic measurements, that cell fluids must be hypertonic to plasma. Opie measured the melting point of frozen pulverized blocks of tissue. However, it took about 60 minutes to reach the end point in Opie's experiments. A considerable proportion of such tissues must have been in the liquid phase by the time the end point was reached. Moreover, tissues thawed from the temperature of liquid N_2 to no more than 0°C. in 1 to 2 minutes will show increases in osmotic activity within 2 to 4 minutes, when kept (between freezing point determinations) at 0°C. Therefore, one would predict a significant increase of solute concentration in the liquid phase of Opie's tissues during the period required for determination of the melting point. Such experimental conditions will not allow a precise determination of osmotic activity of freshly prepared homogenates, so that Opie's results must be excluded as rigorous evidence of intracellular hypertonicity.

The objections to Sabbatani's (15) experimental data are: (1) no special device to homogenize tissues; (2) all tissues were removed after the death of the animal; (3) the time interval between removal of tissue and determination of freezing point depression was from 2 to 60 or more minutes, while the tissue was kept at room temperature. Data reported from this laboratory (45, 46) and elsewhere show that freezing point depression of fresh tissues kept at room temperature can increase over twofold in 20 minutes.

Recent data on swelling of tissue slices in isotonic media have led to the concept of intracellular hypertonicity. The fact that oxygenation of the ambient solution resulted in a decrease in size of swollen slices was considered convincing evidence in favor of hypertonic cell fluid (21, 22). Presumably an active water pumping mechanism maintains an osmotic gradient between cell fluid and surroundings in the living animal. After removal of oxygen, water moves passively in the direction of its chemical potential gradient, thus increasing the volume of cell fluid. However, Deyrup's findings (see above) are difficult to explain by water pumps alone. Many workers (31, 34–39)

have shown that certain ionic fluxes in tissue slices can be related to oxygenation or to swelling of the slice.

Therefore, the volume changes observed in slices immersed in isotonic media may be due to the movement of both solutes and water rather than to the movement of water alone between the slice and the ambient solution. This means that no osmotic gradient need be present to account for the phenomena of swelling or shrinking in the immersed slices.

On the basis of factual evidence to date, we know only that the freezing point depression of tissue homogenates is greater than that of plasma. The basic question of whether or not cell fluid is isotonic to its surroundings has been approached only indirectly (by extrapolation of cryoscopic values or by deductions from tissue slice experiments) and not directly. If cell fluid is isotonic, the problem would be to determine whether tissue homogenates become hypertonic because of physicochemical changes induced during their preparation, or because of metabolic processes active even at 0°C. If cell fluids are hypertonic, the problem would be to determine the precise nature of the water transport mechanism.

SUMMARY

The freezing point depression of freshly excised frozen tissues, pulverized in a hydraulic press or in a mortar, is greater than that of plasma. Even at 0°C. the freezing point depression of such homogenates increases significantly with time. Dilution data indicate that such freezing point data are valid. The presence of intact cells has been shown in smears of tissues pulverized in a mortar, but not in smears of those crushed in a hydraulic press. The osmolarity of various diluent solutions affects the calculated osmotic activity of tissue homogenates presumably because of delayed diffusion between the diluent and cell fluid. With a hypertonic NaCl diluent, spuriously low values of tissue osmotic activity are found from calculations assuming instantaneous mixing between homogenates and diluents. The limitations of data from cryoscopic experiments and from tissue-swelling experiments are discussed in relation to the basic question of whether or not cell fluid is isotonic to extracellular fluid.

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THE RESPONSES OF THE PUPIL OF GEKKO GEKKO TO EXTERNAL LIGHT STIMULUS

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PLATES 1 AND 2

(Received for publication, April 11, 1956)

The eyes of the nocturnal geckos are in one important respect simple and therefore useful for physiological experiment; their retinas have only rods (Walls, 1942; Detwiler, 1943; Rochon-Duvigneaud, 1943) and thus the interpretation of results obtained upon their vision is not made difficult by the uncertainty as to the relative roles of rods and cones. A study of the responses of the pupil of *Gekko gekko* to external light stimulus, in itself a subject of great interest because the gecko's pupil responses are exceptionally efficient ones, is used here to give information about the properties of the gecko's retina.

The gecko's pupil differs markedly from the human in that: the pupil is a slit pupil (Fig. 1) not a circular one; the two irises are completely independent: if one is brightly illuminated and the other in darkness the first pupil will be very tiny whilst the second remains very large; the pupils are under independent voluntary control, the gecko can close a pupil tightly independently of the stimulating light intensity. The last facility is sometimes, but not often, a nuisance during the experiments.

Methods

Fixation of the Eye

The gecko can make only very small movements of the eye within its orbit; the problem of fixing the eye can therefore be reduced to that of keeping the head still. A piece of cylindrical glass 6 cm. in diameter and 28 cm. long was taken; half of the tube was cut away for 6 cm. at one end and the glass replaced by centimeter wire netting. A flat glass plate was placed in the tube to make a platform for the gecko to stand upon and a wedge-shaped piece of cork was placed on top of this platform under the netting with the broader part of the wedge near the end of the tube. When the gecko was placed on the platform at the open end of the tube he ran along to the closed end of the tube, putting his head firmly on the wedge-shaped space formed by the cork, platform, and netting (Fig. 2). A piece of soft rubber foam sheet was pushed behind the gecko to prevent his escaping. Even in this confined space the gecko

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could move and even turn round but it very rarely did so, usually staying quite still (probably shamming dead when the observer was nearby).

The glass tube containing the gecko was held on to the movable block of a Palmer adjustable stand by two curved friction tight springs. The tube could thus be raised or lowered with the block on the stand and turned along its axis in the springs.

Light Sources

Two types of light source were used. The first (type A) was identical with those previously described (Denton and Pirenne, 1954 *a*), a tungsten filament 12 volt, 12 watt opal bulb being the source of light, its intensity being controlled by two neutral wedges and its spectral composition by placing combinations of glass and gelatin colour filters in the light beam. The second source (type B), used for experiments in which a higher intensity of light was required, was made of a tungsten filament 12 volt, 80 watt opal bulb enclosed in a light-tight, water-cooled box, the box being closed by holders for neutral and colour filters. The intensity of the second source could be changed only in steps of about 0.1 log unit by changing the neutral filters.

The light sources were calibrated for brightness and colour temperature and the filters for spectral transmission by the methods previously described (Denton and Pirenne, 1954 *a*).

Methods of Observation

In these experiments visual observations (sometimes through a telescope), which were always used when possible, were supplemented by measurements of photographs of the pupil. Infrared photographs were made using Kodak I.R.E.E. plates; the infrared light for the photographs was given by a source type B, the radiations other than the infrared being filtered out by a Chance OX 5 filter. With only the infrared source, the pupil was shown to have the same area when the distance from the source to the gecko's eye was halved; again, at higher intensities where visual observations could be made, cutting on and off the infrared source had no effect on the pupil. From these observations it was concluded that the infrared light used to take photographs did not significantly change the pupil responses. For the higher intensities of stimulating light photographs were made using Ilford H.P.3 plates.

The eye and pupil of the gecko were so still that exposures of several seconds could be used to take the photographs. The area of the pupil was measured from the photographic plates by projecting a greatly enlarged image of the eye on to graph paper, drawing round the image of the pupil, and counting the squares contained in the outline of this image. This method makes no allowance for corneal refraction and the pupil areas given below are the apparent pupil areas as seen from outside the eye, not the real pupil areas. The photographs were taken along a line which made an angle of 20° with the perpendicular to the gecko's iris.

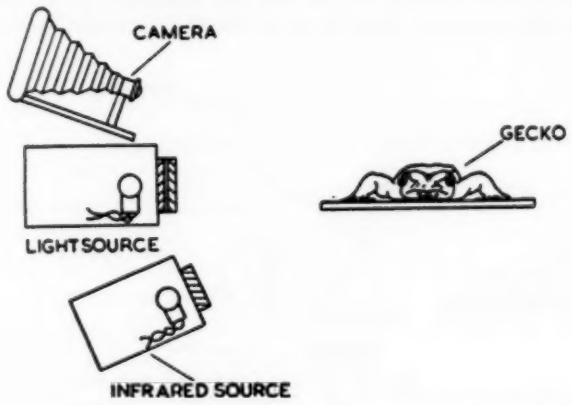
Variation of Pupil Area with Angle of Incidence of the Light

The relative positions of the eye and the light source could be changed in moving the light source and also by changing the position of the tube holding the gecko. Visual observations and photographic measurements of the changes in area of the

pupil with angle of incidence were made for lights differing in spectral composition and in intensity.

Effect of Intensity of Light

Photographic measurements were made of the pupil area for a series of light intensities. The two types of light source were used to give an extended range of intensities. In this experiment the source was placed at a distance such that it subtended 5° at the gecko's eye. These measurements were made only for white light coming to the gecko's eye in a direction perpendicular to the iris.



TEXT-FIG. 1. Diagram of the apparatus used to study the effect of light intensity on the pupil area of the gecko.

The Degree of Spatial Summation of the Response

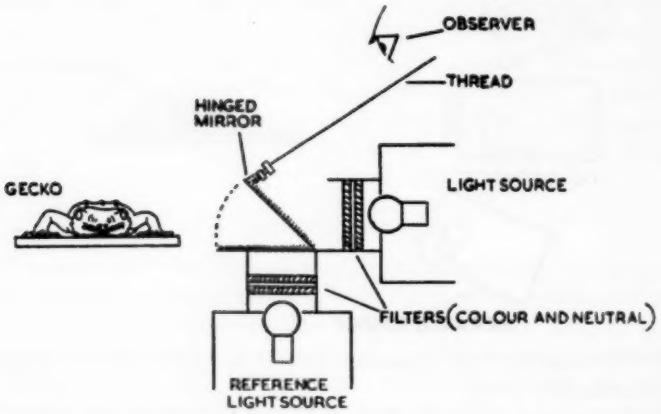
Only two angular fields were used: the source itself at a distance such that it subtended 5° at the gecko's eye and a source subtending 140° formed by placing a circular opal glass between the 5° source and the gecko. A circular opal glass and circular neutral filters could be placed side by side in a slide carrier borrowed from a slide projector. In one position of the slide carrier the gecko saw the light source itself through a neutral filter and subtending 5° at the eye, and in the other the opal illuminated by the same source subtending 140° at the eye. The neutral filter was changed until upon moving the slide carrier from one position to the other there was no change in the size of the pupil. This comparison was made for a number of intensities and colours of light.

The Losses in the Opal.—Two pieces of the flashed opal glass used in the experiment were taken, one larger than the other. In front of the larger opal a large neutral filter density 1.0 was placed. One-half of this neutral filter was covered with the smaller opal and the other by a second neutral filter. This whole assembly was placed perpendicularly between the light source and the eye of the experimenter with the larger opal towards the experimenter. The value of the second neutral filter was changed until the two fields corresponding to this neutral filter and to the smaller

opal appeared equal. The joining edge of these fields was hidden with black paper. The absorption of the "matching" neutral filter, whose density was about 0.4 log unit, was taken as equal to the losses in the opal. In this calibration the conditions are very close to those used in the experiment upon the gecko, the larger opal corresponding to the plane of the gecko's eye. The density 1.0 filter was used to make the effects of successive reflections between the opals unimportant.

The Determination of Spectral Sensitivity

Two light sources of type A were placed perpendicular to each other. A mirror, made of stainless steel polished on one side and blackened on the other, could be moved quickly with a piece of thread between two stops in such a way that one source



TEXT-FIG. 2. Diagram of the apparatus used to measure the spectral sensitivity of the gecko's eye.

could be replaced by the other in the gecko's field of view. In one position, the mirror lay flat, cutting off the horizontal light source and the gecko saw directly the vertical source. In the other position, with the mirror raised to 45°, the vertical light source was cut off from the gecko's eye and the gecko saw the reflection of the horizontal source (Text-fig. 2).

The reference horizontal light source was kept constant in intensity and in spectral composition, the light being that transmitted by a reddish orange combination of the Chance OR 2 filter and the Corning 2412 filter.

The vertical light was varied in spectral composition by placing coloured filters in the light beam. For each combination of filters used the intensity of the source was changed using its neutral wedges until a "match" was made between the two sources, that is, on switching from one source to the other the pupil of the gecko neither constricted nor dilated. The spectral sensitivity of the gecko was determined in this way for wave bands from 370 to 720 mμ. Matching methods such as this one have the advantage that their accuracy depends only upon the constancy of the differential sensitivity and not upon the constancy of the absolute sensitivity of the response.

The intensity chosen for the comparison source was one for which the rate of change of the pupil area with change in intensity was very high. The calibrations of light intensities at the eye were simple because the vertical light source upon which the measurements depended shone directly onto the eye.

Two types of experiments were performed. First the approximate curve was determined by making measurements for all the filters in turn without any allowance for a possible change in differential sensitivity of the retina during the experiment. Such series were made by two observers. The second method was to make successive measurements for a given filter and the filter Ilford 604. In this way each filter is compared in turn against the Ilford 604 and the small effects of change in differential sensitivity during the experiment can thus be almost eliminated.

RESULTS

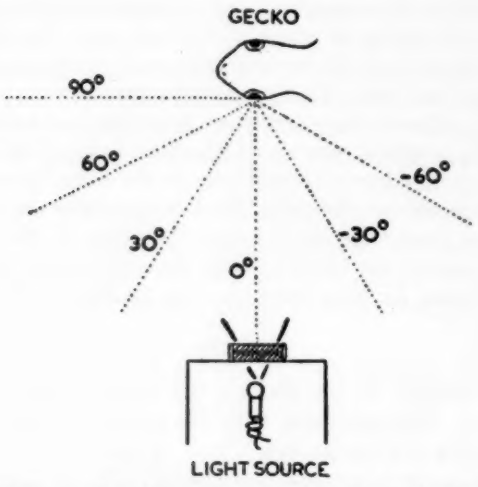
The external diameter of the gecko's eye along its optical axis was estimated to be 1 cm. This compares with the corresponding figure of 2.4 cm. (Le Grand, 1946) for the human eye.

On projecting a small light image onto the gecko's eye and moving it across the eye it was shown that the pupil response was only to light entering the pupil of the eye; no response of the pupil to light falling only on the iris could be seen. It is therefore probably safe to assume that the pupil response of the gecko is mediated by way of the absorption of light by the photosensitive pigments of the retinal receptors.

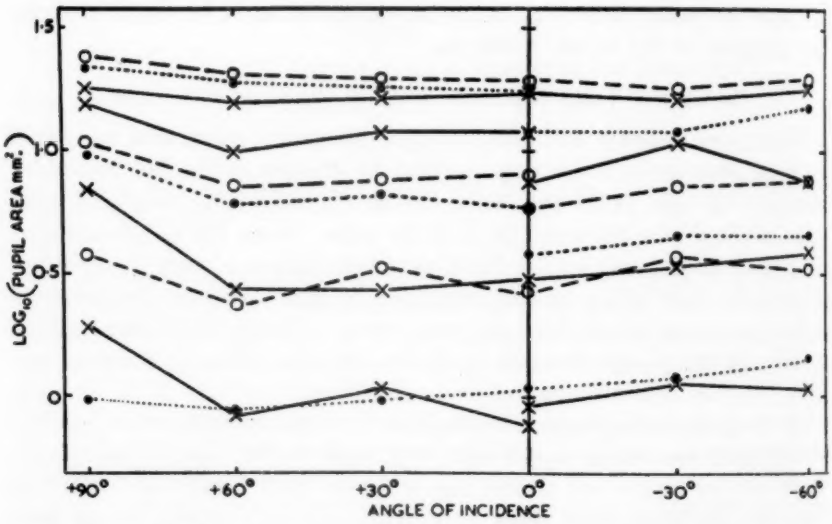
Variation of Pupil Area with Angle of Incidence of the Light

For a given intensity and colour of light the gecko's pupil area was found by visual observation to be very constant for changes in the direction of incidence of the light at the eye; there was no suggestion of a specialized pupil reflex localized in a particular part of the retina. Using the same apparatus the human subject was seen to have, as is well known, a more powerful pupil reflex for light falling on the fovea than for light falling on the periphery of the eye, and an anesthetized cat gave a much stronger pupil reflex for light striking the eye in one direction (probably for light falling on the area centralis) than for light striking it in other directions.

For the gecko photographs were made as the angle of incidence of the stimulating light was varied across one "meridian" in 30° steps (Text-fig. 3). Three spectral bands and several intensities for each band were used. The three spectral bands were those isolated by the red Corning cut off filter 2408, transmitting wave lengths greater than about 620 mμ, the green filter Ilford, 604, maximum transmission about 520 mμ, and the blue filter Corning 5113, maximum transmission about 420 mμ. The results of these measurements are shown in Text-fig. 4. The area of the pupil varied for a given light very little with angle of incidence, the lines showing sensitivity to different colours are seen moreover to be everywhere approximately parallel to one



TEXT-FIG. 3. Diagram of angles used to study the variation of pupil response with angle.

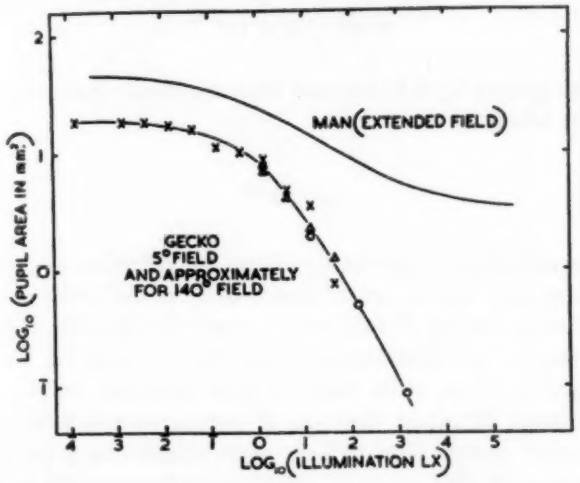


TEXT-FIG. 4. Variations of pupil area with angle of incidence of the source for several different intensity levels for three colour filters. Ilford 604 (λ_{max} = 0.52 μ)—×—×—. Corning 5113 (λ_{max} = 0.42 μ) ....●....●..... Corning 2408 (cut-off filter transmits wave length greater than 0.61 μ)---○---○---. One line across the graph is for a given filter at a given intensity. Several different intensity levels are used for each filter; these intensity levels are spaced approximately 1 log unit apart.

another; this means that if there are changes in relative spectral sensitivity of the pupil reflex across the gecko's retina these changes must be exceedingly small.

Variation of Pupil Area with Intensity of Light

The change of pupil area with light intensity for white light (that of a tungsten filament opal lamp of colour temperature 2400°K.) is shown in Text-fig. 5. The curve of log pupil area against log intensity for the gecko is here



TEXT-FIG. 5. Pupil area against intensity of light. The symbols +, △, and ○ represent different experiments all made with 5° sources. The curve for man is taken from Le Grand (1948) and is for an extended field. The illumination scale at the bottom applies both to man for an extended field and to the gecko for a 5° and approximately for a 140° field.

compared with a similar curve given for man by Le Grand (1948) on the basis of the experimental results of Crawford (1936) and Flamant (1948). The lower limit of pupil area was not reached in these measurements for on holding the gecko with its eye towards a "brightly" lit Aberdeen summer sky the pupil was seen to be smaller in area than the smallest area measured photographically. The smallest pupil seen was made up of four very tiny holes, the largest being estimated, by comparison with holes of known size, to be about $\frac{1}{10}$ mm. in diameter.

The Degree of Summation of the Response

The source itself was 16.5 cm. from the eye; the diffusing surface of the opal (radius 4 cm.), when placed between the source and the gecko's eye,

was 15 cm. from the source and 1.5 cm. from the eye. If such a source has a candle power of L *cd* in the direction of the center of the opal, then the illumination at the center of the opal will be $\frac{L}{(0.15)^2}$ lx. Assuming the opal to be uniformly illuminated (at its edge its illumination will in fact be about 7 per cent less) and that all the light striking the opal is perfectly diffused on the other side in the direction of the gecko's eye, then the illumination at the gecko's eye would be

$$\frac{L}{(0.15)^2} \times \frac{4^2}{4^2 \times 1.5^2} \text{ lux} \qquad \text{(Walsh, 1953).}$$

This would be greater by 0.12 log unit than the direct illumination of the eye by the source which is

$$\frac{L}{(0.165)^2} \text{ lux}$$

The above calculation neglects the losses by reflection and absorption in the opal. These were found (see *Methods*) to be of the order of 0.4 log unit. Thus simply replacing the 5° field of the source by the 140° field of the opal would have reduced the illumination at the eye by about 0.3 log unit.

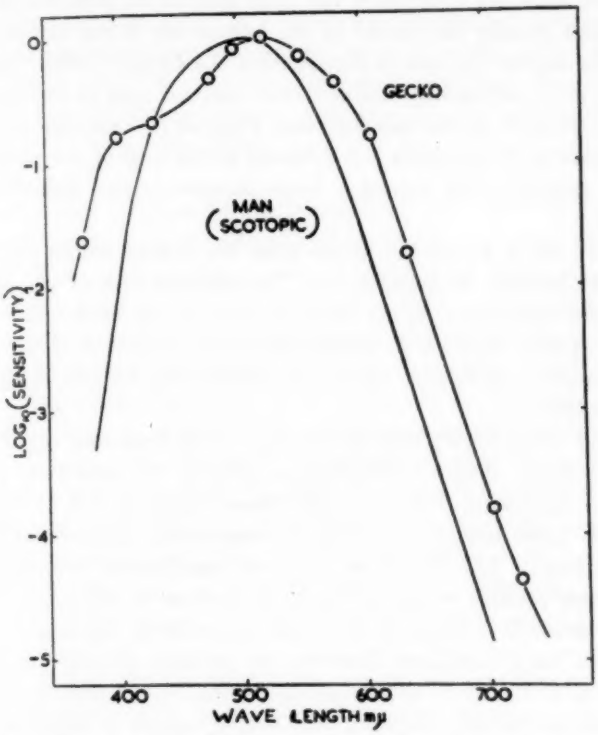
It was found over the whole range of pupil response, within which visual observations could be made, that the 5° source viewed directly through a 0.2 to 0.3 density neutral filter had the same stimulating action on the pupil reflex as a source of 140° made by substituting between the source and the gecko's eye an opal glass for the neutral filter. For a given pupil response the total amount of light flux entering the pupil of the gecko was then 0 to 0.1 log unit more for the 140° source than for the 5° source. The calculations upon which this result depends were simplified by the assumption that the opal perfectly diffused the transmitted light. The method used for finding the losses in the opal glass was, however, one which averaged the transmitted diffused light and the use of the value so given for these losses largely corrects for the errors which the assumption of perfect diffusion would otherwise give. The curve of log pupil area against log light flux entering the eye for a 140° source was then of the same form as the curve for a 5° source, being perhaps a little displaced along the intensity axis.

The Determination of Spectral Sensitivity

Measurements made using the second method were, for this type of experiment, very accurate and the results were always reproducible within a range of 0.1 log unit.

In Text-fig. 6 the spectral sensitivity curve for the gecko is compared with the C.I.E. scotopic sensitivity curve for man (C.I.E., 1951). It can be seen

that the gecko's curve is similar in shape to the human scotopic curve but is displaced between 20 to 30 mμ towards the longer wave length end of the spectrum, this displacement appearing greater for the red end of the curves than around the maxima. The latter fact may be caused by a higher density of photosensitive pigment in the gecko's rod than in human rods.



TEXT-FIG. 6. The spectral sensitivity curve of the gecko as compared with the C.I.E. scotopic curve for man. The ordinate is of log sensitivity; a constant has been arbitrarily added to the ordinate values to make the maximum sensitivities equal to unity.

In the near ultraviolet the gecko appears to be more sensitive than the human subject. There is, however, some uncertainty about the meaning of the value given for $\lambda = 370$ mμ because the crystalline lens gave a bluish green fluorescence for light of this wave length. This is a well known effect for other vertebrate lenses and Le Grand (1938) has measured the spectral composition of the light given by such a fluorescence. If the light given by the fluorescence of the lens were the effective stimulus to the pupil response the direct sensitivity to the near ultraviolet must be even less than that shown in Text-fig. 6.

DISCUSSION

In many of the arguments which follow the assumption is made that the pupil response, which depends upon light entering the pupil, is mediated by the retinal receptors.

The eye of the gecko has two features of special interest: one of the most efficient irises found in nature and the large rods of its pure rod retina.

The function usually attributed to the human iris is one of protecting the retina against sudden changes in illumination (Le Grand, 1948). This function is, however, very inefficiently fulfilled; the minimal area of the human pupil is only one-sixteenth of the maximal and, even at its best, the response to a tenfold increase in illumination is a decrease in the area of the pupil to three-fifths of its original value leaving a large increase (about sixfold) in retinal illumination.

The gecko's iris is altogether better than the human iris in protecting the retina against increase in illumination. The minimal area of the pupil is less than one-three hundredth of the maximal and, at its most efficient, the response to a tenfold increase in illumination is a reduction of the pupil area to about one-sixth its former value, the retinal illumination being therefore not even doubled.

Aguilar and Stiles (1954) have shown by a most ingenious experiment that "at a field intensity of about 100 scotopic trolands the sensitivity of the (human) rod mechanism to stimulus differences begins to fall off rapidly and that at 2,000–5,000 scotopic trolands (corresponding approximately to daylight luminances of 120–300 cd/m^2) the rod mechanism becomes saturated and is no longer capable of responding to an increase in stimulus." (100 cd/m^2 is the illumination in a room lit by moderate artificial lighting.)

The gecko's pupil continues, however, to contract sharply for an increase in intensity of a 5° source of luminance 100,000 cd/m^2 and since the gecko has only rods as retinal receptors this source, which is about 1,000 times brighter than that required to "saturate" completely the human scotopic visual system, gives a retinal illumination insufficient to saturate the gecko's rod visual system. The gecko's eye, lacking cones, is clearly adapted to allow the rods to be useful for vision at much higher external illuminations than are the human rods. Part, but by no means all, of this adaptation is the reduction of the intensity of light reaching the retina by a very efficient pupil.

The retinal illumination is given for the human eye by the formula $D = 0.36\ B.S.\tau_\lambda$ (Le Grand, 1948) in which D is the retinal illumination in lux, S the area of the pupil in cm.^{2}, B the brightness of the external source in cd/m^2, τ_λ the transmission of the ocular media.

Taking account of the difference in size of the human and gecko's eye, the formula $D = 0.36\ B.S.\tau_\lambda$ for the human eye becomes approximately $D =$

2.25 $B.S.\tau_\lambda$ for the gecko. For $B = 10^5$ cd/m^2, S is for a 5° source, 9.8×10^{-4} cm^2. Taking $\tau_\lambda = 1$, $D = 2 \times 10^2$ lux.

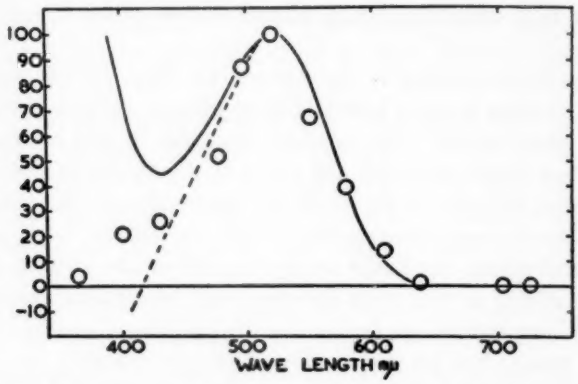
The gecko's rod has a cross-section about 16 times that of the human rod and it can be calculated as described by Denton and Pirenne (1954 *b*) that the gecko's rod is not saturated when approximately 10^6 quanta/sec. of the wave length 520 mμ fall upon it. Unpublished measurements (Denton) upon two other species of gecko gave for $\lambda = 530$ mμ densities of about 0.5 for the photosensitive pigment *in situ*; this means that of the light of this wavelength striking the retina about 70 per cent is absorbed. We have then the remarkable fact that the gecko's rod, which probably like the human responds to the absorption of 1 quantum, is not saturated even when absorbing almost 10^6 quanta/sec.

A more important function of the human iris than that of shielding the retina against changes in light intensity is to give to the pupil the optimum diameter for visual acuity. The minimal diameter of the human pupil of about 2 mm. is a value for which the limits to acuity set by the fineness of the retinal mosaic and size of the pupil are approximately the same (Shlaer, 1937). When the external illumination is high the human pupil is made as small as is useful within the limits set by the diffraction of light in order to cut down the effects of the eye's spherical and chromatic aberrations. At lower levels of illumination the acuity is probably limited not by the fineness of the retinal mosaic but by the number of quanta reaching the retina (Pirenne and Denton, 1952) and it is then useful to increase this number by increasing the pupil area even though the distortions caused by optical defects of the refractive system of the eye will become more disturbing.

For man then the minimal pupil diameter of approximately 2 mm. and a retinal mosaic of cones 2 to 2.5 μ apart (Pirenne, 1948) set approximately the same limit to acuity (Shlaer, 1937). The gecko has a minimal pupil diameter of about 0.1 mm. Such a pupil will probably set the same diffraction limit to angular acuity as would a 0.1 mm. pupil in man, that is 14 times that of man with a 2 mm. pupil (Lister, 1913). Again for the gecko a retinal mosaic of rods 10 μ apart (Detwiler, 1943) in an eye of external diameter two-fifths the dimension of the human eye gives a limit to angular acuity 12 times as great as does the human retinal mosaic. For the gecko the two limits to acuity may not therefore be of exactly the same value, but they are nevertheless still close to one another and for the gecko's eye we see almost the same elegant economy in design as for the human eye.

For the human eye external objects situated at a distance for which the eye is not accommodated will be blurred. An example taken from Listing (1851) is that for a circular pupil of 4 mm. diameter and an eye accommodated for infinity a point object at 9.4 cm. from the human eye will give not a point retinal image but a circular retinal image (circle of diffusion) of di-

ameter 0.58 mm., corresponding to a circular external field of diameter 2°. For the gecko when the pupil is a narrow slit the retinal images of external objects for which the eye is not accommodated will be sharper horizontally than vertically; when its pupil consists of a vertical series of holes, such an object will give a series of retinal images, one for each hole, displaced vertically from one another. (This effect is seen for the human eye in the well known experiment of Scheiner.) The gecko with an eye two-fifths the linear dimension of the human eye had for the higher external experimental illuminations used a pupil made up of a series of holes spaced along a 4 mm.



TEXT-FIG. 7. Comparison of the absorption spectra of extracts from the retinae of *Phyllurus milii* (Crescitelli) and the spectral sensitivity curve (plotted on a quantum basis) of *Gekko gekko*. Solid line, relatively pure unbleached spectrum and broken line, NH_2OH difference spectrum of *Phyllurus milii*; open circles, sensitivity on a quantum basis of *Gekko gekko*. Each curve is scaled so that its maximum is 100 per cent.

line, and hence in the conditions described above, the circular retinal image corresponding to 2° for the human becomes a series of retinal images corresponding to an arc of approximately 5° in the external field.

Dr. F. W. Campbell (private communication) has suggested that the gecko might use this effect to judge the distance of external objects either by finding what degree of accommodation is required to bring the vertically spaced images into coincidence or that "the gecko might for example wait until the 4 flies coming toward it become one fly and then bite."

Unless the absorption spectrum of the ocular media is very anomalous, the rod vision of *Gekko gekko* is mediated by a photosensitive pigment similar to rhodopsin but with its maximum displaced between 20 and 30 mμ towards the red end of the spectrum. (The lenses of other geckos examined were quite colourless.) This conclusion is confirmed by the careful measurements of Crescitelli (1956) upon several species of geckos. He found in particular that the

photosensitive pigment extracted from a closely related species of gecko, *Phyllurus milii*, has an absorption curve similar to that of rhodopsin but its maximum is displaced to 524 mμ. The present measurements of spectral sensitivity when plotted on a quantum basis (Dartnall and Goodeve, 1937) agree well with his results (Text-fig. 7). Again, difference curves measured upon the fresh retinae of two Egyptian geckos, *Tarentola annularis* and *Ptyodactylus hasselquistii*, had maxima displaced about 30 mμ towards the long wave length end of the spectrum with respect to the corresponding maxima for the frog and salamander (Denton, unpublished observations, using a method similar to that described by Denton and Wyllie (1955)).

The geckos seem then to be exceptions to the general rule given by Wald (1945 *a*) that all terrestrial animals have a scotopic vision mediated by rhodopsin. Wald noted, however, that there was then no information on the rod vision of reptiles. The biological significance of these results is discussed by Crescitelli (1956).

The measurements of absorption obtained upon extracted pigments are very precise around the wave length of maximum absorption but become inaccurate when the absolute absorption becomes low; there is also a difficulty in interpreting the results for the shorter wave lengths because the products of bleaching are not colourless. Measurements such as those described here, although they are less precise than measurements upon pigments in solution around the maximum of absorption, are equally precise for every wave band used and their chief interest lies in the information they give for wave lengths at the extremities of the visual spectrum.

The logarithm of the spectral sensitivity of the gecko, plotted on a quantum basis against the reciprocal wave length $1/\lambda$, falls approximately linearly in the long wave end of the spectrum as λ increases. A similar fall is observed for the human rod and cone systems (Stiles, 1948) and *Xenopus* (Denton and Pirenne, 1954 *b*). A theoretical interpretation of the shape of the sensitivity curves in this region is given by Stiles (1948) and Lewis (1955). It is an interesting example of how biological measurements may give information on a physicochemical problem inaccessible to conventional physical chemical experimental methods.

The fall in sensitivity may be described (Stiles, 1948) by equations of the type

$$\log_e \frac{h\nu}{W_\lambda \tau_\lambda} = \text{a constant} + K \frac{h\nu}{kT}$$

in which W_λ is the threshold energy entering the eye for radiation of wave length λ (frequency ν) measured at the cornea of the eye; τ_λ is the spectral transmission of the eye media between cornea and retina, h is Planck's constant, k is Boltzmann's constant, T the absolute temperature (here 291°K.).

K is a constant depending upon the particular photosensitive pigment mediating vision. K, which is 0.79 for human rod vision, 0.87 for human cone vision, 0.75 for *Xenopus*, is found here to be 0.76 for the gecko.

The sudden drop in sensitivity found in the near ultraviolet is probably principally caused by a sudden decrease in the transmission of the crystalline lens. This is the explanation of the similar fall found in man (Goodeve, Lythgoe, and Schneider, 1942; Wald, 1945 *b*). Denton (1955, 1956) has shown that for 27 species of fish, the frog, and the squid, the fall in transmission of the lens on moving towards the shorter wave length end of the spectrum is always sharp but that the wave length around which the fall takes place varies greatly from one species to another. This wave length varies from 440 mμ for *Chlorophthalmus agassizii* to 310 mμ for *Anguilla vulgaris*. Here for the gecko there is probably such a fall in transmission around 370 mμ.

SUMMARY

1. The responses of the pupil of a nocturnal gecko (*Gekko gekko*) to external light stimulus were studied.

2. The responses of the pupil are determined by light entering the pupil and not by light acting directly on the iris.

3. The responses of the pupil are very uniform in sensitivity including spectral sensitivity for light coming in different directions to the eye.

4. The possible change in area of the pupil is more than 300-fold and probably represents an effort to shield the pure rod retina from saturating light intensities.

5. The pupil continues to contract sharply for changes in external light intensity which give retinal illuminations corresponding to 10^6 quanta/sec. striking a retinal rod.

6. There is a large degree of spatial summation of the response; circular external light fields subtending 5 and 140° giving the same illumination at the pupil give approximately the same pupil response.

7. The spectral sensitivity curve agrees with the absorption curve of an extracted pigment from a closely related gecko described by Crescitelli in the followig paper. It is similar to the human scotopic curve but its maximum is displaced about 20 to 30 mμ towards the red end of the spectrum. The fall in sensitivity towards the red end of the spectrum is described by the equation

$$\log_e \frac{h\nu}{W_\lambda \tau_\lambda} = \text{a constant} + 0.76 \frac{h\nu}{kT}$$

I wish to thank Mr. J. Lester, Curator of the Reptile House, Regent's Park Zoological Gardens, London, for the loan of the *Gekko gekko*, Professor A. Hassan of

Ein Shams University, Cairo, for the gift of Egyptian geckos, and Dr. M. H. Pirenne for the loan of colour filters bought with a grant from the Medical Research Council. I am grateful to Mr. A. E. A. McLeod for excellent technical assistance.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Photographs of the eye of the gecko for six different intensities of stimulating light (5° source). These intensities increased from a to f. Photographs a, b, and c were made using infrared-sensitive plates, d, e, and f were made using panchromatic plates.

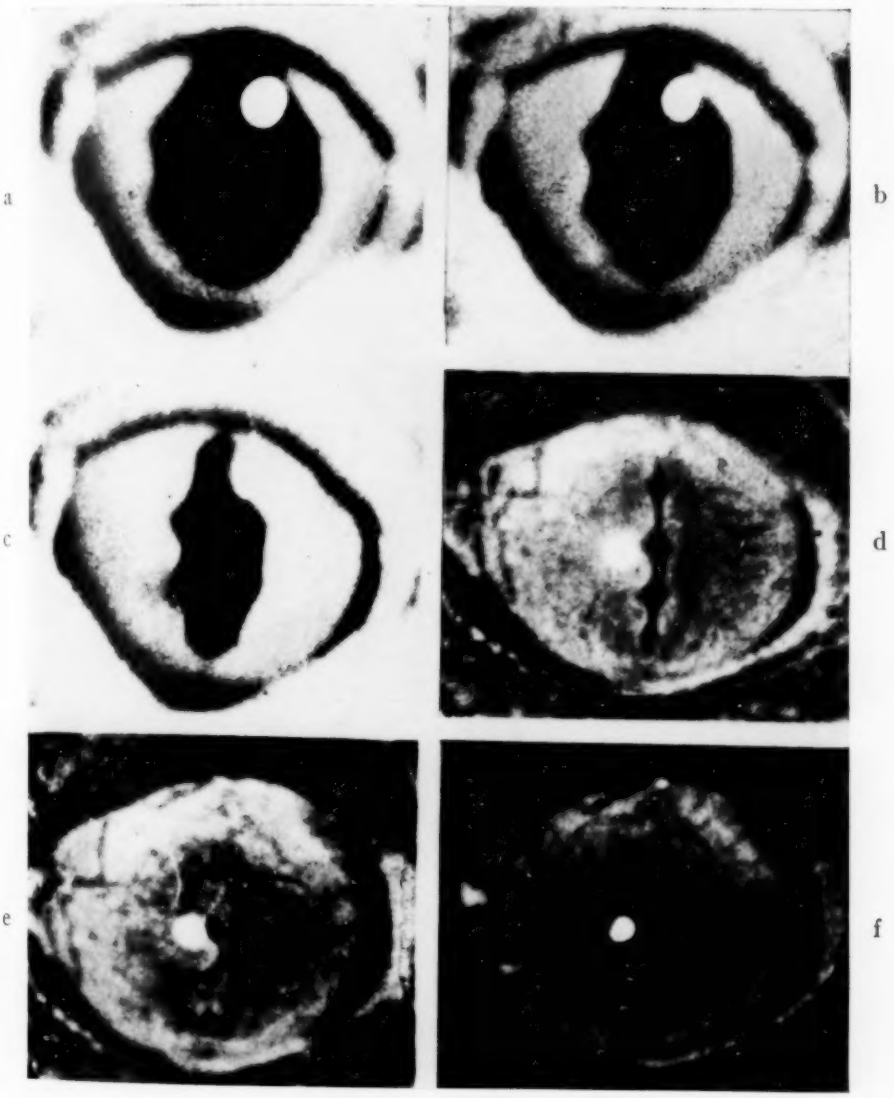

FIG. 1

PLATE 2

FIG. 2. The gecko in position for the experiment.

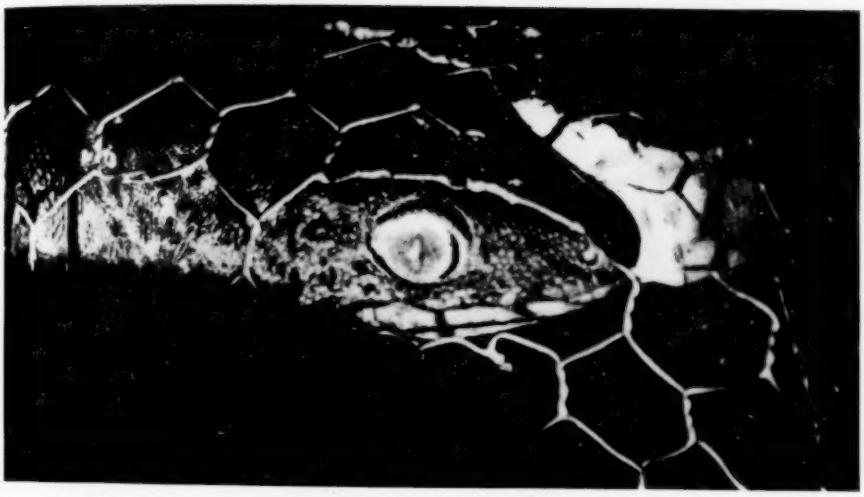

FIG. 2

(Denton: Pupil responses of *Gekko gekko*)

THE NATURE OF THE GECKO VISUAL PIGMENT*

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(Received for publication, April 11, 1956)

The scotopic visual pigments of terrestrial animals are, like the corresponding pigment of the human retina, generally regarded as members of the rhodopsin system; *i.e.*, a group of chromoproteins possessing a characteristic spectrum with an absorption maximum at about 500 mμ and containing retinene$_1$ as a chromophore. It is expected, therefore, that geckos would possess the typical terrestrial type of visual pigment. Recently the spectral sensitivity function of one of these lizards was determined by Denton (1). He reported this curve to be displaced, by about 20 mμ, toward the red end from the position of the human aphakic sensitivity curve. This result means, either that the gecko visual pigment is a rhodopsin and that the preretinal media selectively modify the transmitted light or, alternatively, that the visual pigment is not a rhodopsin (or not a rhodopsin alone). This investigation was initiated as an attempt to ascertain the reason for the unusual sensitivity function of this lizard.

It has been commonly assumed in most of the literature dealing with the gecko visual system that rhodopsin is the photopigment of the retinal rods. Detwiler (2) implied this in his paper on the retina of *Gekko swinhonis* Guenther. Walls (3, 4) employed the word rhodopsin in writing about the pigment of the gecko rods but it is abundantly clear that he did not view this protein as a definite chemical entity but rather visualized a variety of rhodopsin pigments slightly different in different vertebrates. Underwood (5) considered the presence of rhodopsin in the rods as a feature of gecko ophthalmology. In spite of all this, there is nothing in the recent literature on the biochemical nature of this chromoprotein for the case of the geckos. Two older papers cited by A. C. Krause (6) which this writer has not seen, are apparently concerned with the gecko visual pigment. The first is by W. Krause, published in 1893 and the second is by Köttgen and Abelsdorff, published in 1895. A. C. Krause (6) listed 500 mμ as the spectral maximum for the gecko pigment examined by Köttgen and Abelsdorff but in view of the methods that were available in the year 1895 such a figure requires confirmation.

*Aided by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, United States Public Health Service, and by a grant from the University Board of Research.

The present investigation involved the extraction of the photosensitive pigments from the retinae of several species of geckos, the determination of the absorption spectra of these pigments, and the comparison of the absorption curves with Denton's sensitivity function. For the study, seven species of geckos, all nocturnal, were employed.[1] As yet it has not been possible to obtain *Gekko gekko*, the species used by Denton in his work. In this report the specific details will be given for the case of only one of the seven species; *i.e.*, *Phyllurus milii* (White) from the Warumbungle Mountains, New South Wales, Australia. This course was adopted in the interest of economy and is justified by the following considerations: (*a*) *Phyllurus milii* (White) is a member of the same family—the Gekkonidae—as is *Gekko gekko*; (*b*) retinal extracts from *Phyllurus* were purer than the others; and (*c*) these extracts contained the greatest concentration of photosensitive pigment. These last two characteristics made it possible to obtain the most precise data for the case of *Phyllurus*. In any case, the results obtained with this gecko are in no way strikingly different or unique. As a tentative generalization, and within the framework of ideas to be presented, the present investigation describes the main scotopic pigment of nocturnal geckos.

Analytical Procedure

The apparatus and methods were identical with those previously employed for the analysis of the lamprey pigment (7). After dark-adapting the animals for at least an hour, the heads were removed and the eyes were excised and placed in 4 per cent potassium alum for 30 to 60 minutes. The retinae were extruded through an opening in the cornea and were then placed in the alum solution for 16 to 20 hours. The hardened tissue was then washed twice with distilled water and once with borate–KCl buffer (pH = 8.3). The photosensitive pigment was then extracted from the retinae with 2 per cent digitonin made up in the alkaline borate–KCl buffer. The volume of digitonin solution varied in separate experiments from 0.5 to 1.5 ml. depending on the amount of retinal tissue which was available. The complete extraction was accomplished in two successive steps, employing two-thirds of the total volume of digitonin solution in the initial step. All these procedures were carried out in a dark room, using for illumination a deep red photographic safe light.

Since such extracts are likely to be somewhat unstable for a few days after preparation, the usual procedure was to store them in a refrigerator at about 10°C. for 1 to 3 weeks before analysis. At convenient intervals 0.5 ml. of the extract was transferred to a microcell and optical density measurements were made from 700 to 340

[1] The author is greatly indebted to the following for providing some of the animals: Mr. S. Kellner and Mr. H. G. Cogger, Sydney, Australia; Dr. P. Tardent and Dr. P. Dohrn, Stazione Zoologica, Naples, Italy; Dr. R. B. Cowles, Mr. B. Brattstrom, Mr. J. Cunningham, and Mr. D. Belkin all of Los Angeles. The author is especially grateful to Dr. G. L. Walls and to Mr. G. Underwood for a stimulating correspondence on the subject of this investigation.

mμ. For these measurements, made with the extract at 20 ± 0.5°C., a Beckman DU spectrophotometer with a photomultiplier attachment was employed. Data for the absorption spectrum of each unbleached extract were first collected following the schedule given previously (7). Following this, the microcell with the extract was then transferred to a bleaching chamber, also at 20°C., in which the total volume of extract was illuminated with colored light provided by a B and L grating monochromator. To eliminate any possibility of contamination by the second order spectrum, interference filters were also used. These were placed between the exit slit of the monochromator and the entrance slit of the bleaching chamber. Colored light was employed as routine in these analyses in order to determine whether or not each extract was homogeneous with respect to photosensitive components. For a complete analysis the typical schedule of bleaching was as follows: (*a*) an initial exposure to red light (660 mμ or longer) followed by a second series of density measurements, (*b*) a second exposure to light of slightly shorter wave length (640 mμ) again followed by density measurements, (*c*) a repetition of the exposure measurement sequence using light of still shorter wave length (606 mμ). The final bleach was of sufficiently long duration to remove all remnants of the photolabile pigment. Variations from the above schedule were occasionally required in order to meet the conditions of particular extracts. Applying this procedure, and using the present equipment, it has been possible to resolve prepared mixtures of rhodopsin and porphyropsin and, in addition, to identify both of these photopigments in extracts of the same retina, in several species (data to be published).

Following the final step in the schedule listed above, the extract was usually exposed to white light or to colored light of wave length much shorter than 606 mμ. Selective density losses as the result of such an exposure do not necessarily indicate the presence of an additional photosensitive pigment in the original unbleached extract. Exposure of a bleached visual pigment solution to light containing shorter wave lengths may result in isomerization of the products of the previous bleaching (8) leading to selective density changes. One way to reduce or to eliminate isomerization is to employ a carbonyl-trapping reagent such as hydroxylamine (NH_2OH) which of itself does not destroy the visual pigment (9). For this, and for other reasons given later, each analysis of an extract contained at least one experiment with added NH_2OH as a component.

RESULTS

A. Absorption Spectrum of the Unbleached Phyllurus Extract.—A relatively pure retinal extract from this gecko yielded, before bleaching, an absorption spectrum which is typical of solutions of visual pigments (curve 1, Fig. 1). This curve is characterized by an absorption maximum at 522 mμ, an absorption minimum at 432 mμ, and a ratio, density minimum/density maximum of about 0.50. Assuming, as will later be shown to be true, that this extract contained only one photosensitive component, the position of the maximum at 522 mμ is very close to, but not identical with, the true peak of the pure pigment. On the basis of the relationship between the density

minimum/density maximum values of retinal extracts and the displacement of their absorption maxima from the true peaks of the visual pigments (10), the true peak of the *Phyllurus* pigment should be close to 524 mμ. This

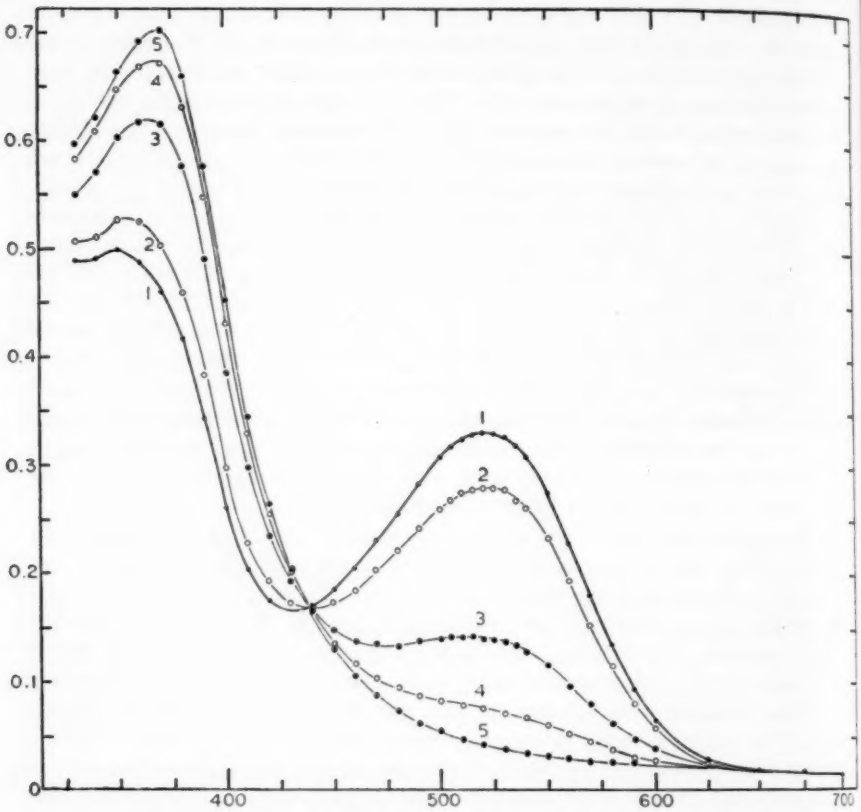

FIG. 1. Absorption spectrum of an unbleached *Phyllurus* extract (curve 1) and the results of bleaching with light at (*a*) 660 mμ for 135 minutes (curve 2); (*b*) 640 mμ for 85 minutes (curve 3); (*c*) 640 mμ for 105 minutes (curve 4); and (*d*) 606 mμ for 25 minutes (curve 5). Optical density is plotted as a function of wave length (mμ). Temperature was 20°C. The pH of the extract was 8.4.

is quite obviously not a rhodopsin. Human visual purple is located at about 497 mμ (11); frog rhodopsin at about 502 mμ (12). A pigment with a peak at 524 mμ and present in the retina of a terrestrial animal is sufficiently unusual to merit further inquiry.

B. Bleaching with Non-Isomerizing Light.—The *Phyllurus* pigment is bleached by light in a manner characteristic of the visual pigments. The spectral changes which resulted from a series of successive exposures to light

of different wave lengths (Fig. 1) reveal the following features: a constant cross-over point at about 438 mμ above which the density decreased and below which it increased. The results suggest the disappearance of a photosensitive pigment and the appearance of a yellow product. This is in accord with the usual behavior of visual pigments in response to illumination (12–14).

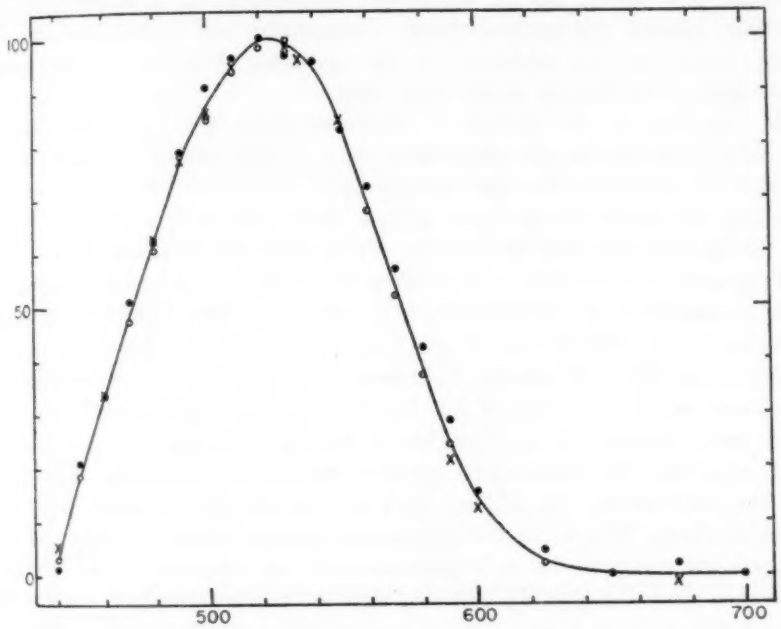

FIG. 2. Difference spectra obtained after bleaching with light at (*a*) 660 mμ (filled circles); (*b*) 640 mμ (open circles); and (*c*) 606 mμ (×'s). Each set of data represents the mean of two separate experiments. The line is the mean curve for all six experiments. The loss in density, as the per cent of the maximum, is plotted as a function of wave length.

The bleaching experiments also permit the conclusion that only one photosensitive component was present in the *Phyllurus* extracts. This statement is based on a comparison of the alkaline difference spectra obtained as a result of the several bleachings (Fig. 2). These spectral density changes indicate a striking uniformity of response, one curve, in fact, representing the density losses in response to each of the wave lengths. This curve is characterized by a maximum loss of density at about 524 mμ. The conclusion that the *Phyllurus* pigment is not a rhodopsin rests, therefore, not only on the spectral position of the absorption curve of the unbleached extract (curve 1,

Fig. 1) but also on the position of the alkaline difference spectrum (Fig. 2). The two methods of analysis yielded identical results.

C. Bleaching with Isomerizing Light.—A 10 minute exposure to white light following the final bleach with non-isomerizing colored light caused a small and selective loss in density, maximal at about 423 mμ (curve 2, Fig. 3). It is doubtful, as the results of the hydroxylamine experiment will indicate, whether this change can be interpreted as evidence of a violet-absorbing pigment. Instead this spectral change was probably the result of an isomerizing action on the products of the preceding bleaches by the short wave length components of the white light.

D. Bleaching in the Presence of Hydroxylamine.—There are two reasons for using NH_2OH in experiments designed to analyze retinal extracts. One—to test for isomerization—has already been discussed. The second reason involves the nature of difference spectra. Since such spectra are determined by the spectral properties both of the products of bleaching and of the original pigment, it is necessary, if the difference spectrum is to be informative, to reduce absorption by the products to a minimum. This, in fact, is the contribution of the NH_2OH technique. Curve 1 (Fig. 3) is an example of an hydroxylamine difference spectrum obtained as a result of a total bleach of a *Phyllurus* extract with light of 606 mμ. This curve is characterized by a distinct break between the positive (loss in density) and negative (gain in density) segments. The positive and negative segments have maxima at 524 and 369 mμ, respectively. The 524 mμ peak is in accord with the peak of the absorption curve (Fig. 1) of the unbleached extract (when corrected, as explained, for the presence of impurities) and with the peak of the alkaline difference spectrum (Fig. 2). The fact that the maxima of the two difference spectra agree, is excellent confirmation of the view that the products of bleaching did not significantly determine the long wave length portion of the difference spectra and that 524 mμ is the true maximum of the visual pigment. This follows logically from the fact that the 524 mμ peak was also obtained when NH_2OH was employed, in which case the oxime which was formed has an absorption peak shifted toward shorter wave lengths. This shift was readily revealed by a comparison of the peaks of the negative segments of the difference spectra with and without NH_2OH. These peaks were at 369 mμ (with NH_2OH) and at 377 mμ (without NH_2OH). Moreover, the cross-over points for the two experiments were at 416 mμ (with NH_2OH) and at 438 mμ (without NH_2OH). Clearly, the NH_2OH resulted in a distinct spectral separation of the product and the original photolabile pigment. This effect is clearly understandable in terms of a reaction of NH_2OH with a retinene to form the corresponding oxime.

The NH_2OH experiment also yielded evidence that the selective spectral change following the terminal exposure to white light in the experiment cited

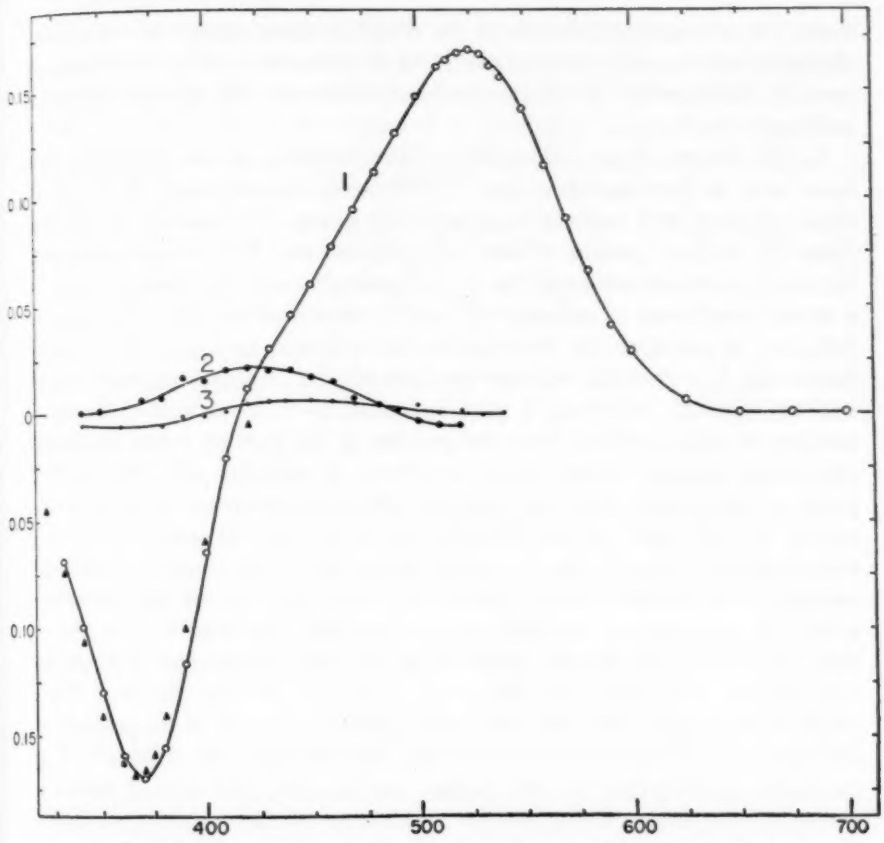

FIG. 3. The NH_2OH difference spectrum following a total bleach with light at 606 mμ (curve 1). Curve 2 is the alkaline difference spectrum (without NH_2OH) obtained after bleaching with white light following total bleach of the extract with red light. Curve 3 is another experiment similar to that represented by curve 2 except for the presence of NH_2OH in the extract. Ordinate values above the zero indicate loss of density; value below zero indicate gain of density. The filled triangles are the spectral densities for a digitonin solution (pH = 8.3) of crystalline all-trans retinene$_1$ to which was added NH_2OH to yield the oxime. These points were scaled for plotting with the difference spectrum.

previously (curve 2, Fig. 3) was probably the result of isomerization. The presence of NH_2OH in the extract almost entirely abolished the white light effect (curve 3, Fig. 3). In view of this no serious defense can be made of the idea that a violet-absorbing pigment was present in the *Phyllurus* extract.

To summarize the preceding sections, it appears that only one photosensitive pigment was present as a significant component of the *Phyllurus* ex-

tract. The absorption spectrum of the relatively pure extract as well as the difference spectra with and without NH_2OH agree in showing that this pigment is characterized by an absorption maximum at 524 mμ and is, therefore, not a rhodopsin.

E. The Nature of the Chromophore.—The evidence of the preceding sections leads to the conclusion that the *Phyllurus* chromoprotein is a typical visual pigment with retinene as a prosthetic group. The position at 524 mμ raises the obvious question whether this pigment may be a porphyropsin and therefore, a remarkable exception to the generalization that porphyropsin is a retinal constituent of animals with a fresh water habitat (15). The original definition of porphyropsin (16) requires that retinene$_2$ be a part of the chromoprotein. It is doubtful whether the data on the *Phyllurus* pigment would conform with this definition. A good indication as to the nature of the chromophore is often obtained from the position of the product peaks (indicator yellow and retinene oxime) under conditions of constant pH. The product peaks as determined from the negative difference spectrum occur approximately at 377 mμ without NH_2OH (curve 2, Fig. 4) and at 369 mμ with NH_2OH (curve 1, Fig. 3). These figures are to be compared with the corresponding average product peaks—377 and 369 mμ—of the rhodopsin system of a number of Amphibia which have been examined in this laboratory. In contrast, the product peaks of the porphyropsin system in Amphibia (unpublished data from this laboratory) occur at 397 and 383 mμ. These comparisons suggest that the *Phyllurus* pigment, in spite of its position at 524 mμ, contains as a carotenoid moiety, not retinene$_2$ but retinene$_1$. This conclusion is reinforced by two further comparisons. The first of these involves a comparison, under similar conditions, of the negative difference spectrum in the NH_2OH experiment with the absorption spectrum of a retinene$_1$ oxime (Fig. 3). The retinene$_1$ oxime was prepared by adding to a solution of the crystalline all-trans retinene$_1$ in 2 per cent digitonin (pH = 8.3) a small volume of freshly neutralized NH_2OH.[2] The absorption curve of this oxime (filled-in triangles), when corrected to scale with the difference spectrum, strongly suggests that retinene$_1$ oxime was in fact the product of bleaching of the *Phyllurus* pigment in the NH_2OH experiment. In the second comparison (Fig. 4) the alkaline difference spectra obtained after bleaching of a rhodopsin solution (curve 1), a porphyropsin solution (curve 3), and the *Phyllurus* extract (curve 2) are shown plotted together on a comparable scale. It is clear that, whereas most of the positive section of the *Phyllurus* difference spectrum is similar to the corresponding portion of the porphyropsin, the negative section, which is related to the products of bleaching, resembles the corresponding section of the rhodopsin. The *Phyllurus* chromo-

[2] The crystalline retinene was kindly provided by Dr. Grove Baxter of Distillation Products Industries.

protein is clearly not a porphyropsin but a member of the retinene$_1$ class of photopigments. The presence of retinene$_1$ as a chromophore of the *Phyllurus* pigment accounts for one result which is usually not obtained in work on visual pigments. This is the agreement in position of the peaks of the differ-

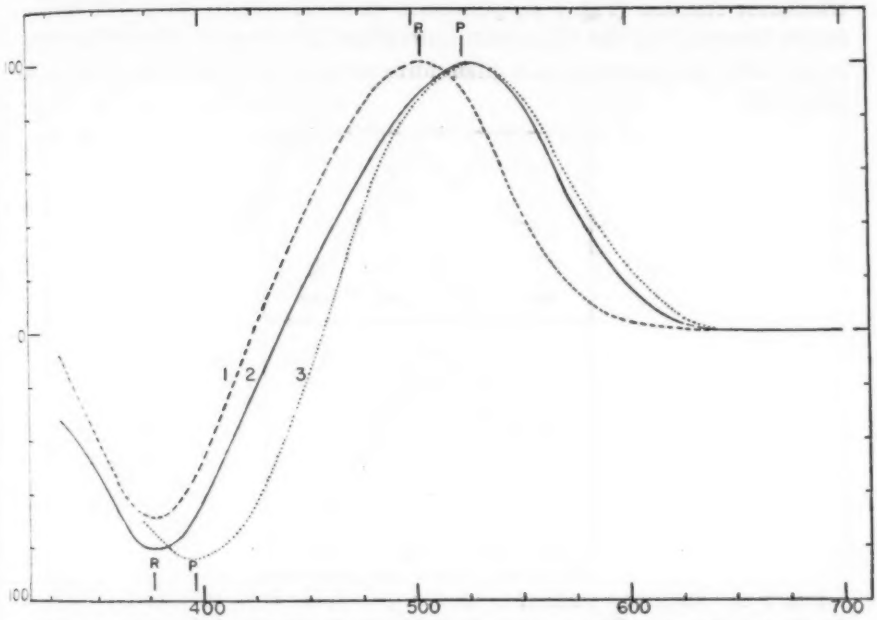

FIG. 4. A comparison of the alkaline difference spectra obtained with a rhodopsin extract (curve 1); a porphyropsin extract (curve 3); and the *Phyllurus* pigment (curve 2). The rhodopsin and porphyropsin extracts were prepared from the retinae of the alligator and carp, respectively. Density change as per cent of the maximum loss is plotted as a function of wave length. Values above the zero ordinate indicate loss of density; values below zero indicate gain of density. The conditions were identical for all three extracts. The short vertical lines represent the mean positions of maxima for density loss and for density gain for amphibian rhodopsins (*R*) and amphibian porphyropsins (*P*).

ence spectra with and without NH_2OH. This agreement is undoubtedly due to the large spectral separation which already exists between the gecko pigment (at 524 mμ) and retinene$_1$ indicator yellow (at 377 mμ).

As an additional reinforcement of this general argument, it should be pointed out that biochemical agreement exists between the retina and the liver. This point has been carefully tested, employing the liver and retinal systems of the banded gecko, *Coleonyx variegatus*. Using the same methods and arguments which were applied to the analysis of the *Phyllurus* pigment,

it was found that *Coleonyx* possesses a retinal photosensitive pigment with a maximum at about 520 mμ and that the product of bleaching of this pigment is retinene$_1$. The liver of this lizard was found to contain a quantity of vitamin A_1 but no vitamin A_2. This fact is demonstrated by the antimony-trichloride reaction (Fig. 5 A) performed on a chloroform extract of the *Coleonyx* liver and by the ultraviolet absorption spectrum of this extract compared with the spectrum of a chloroform solution of crystalline vitamin A_1 (Fig. 5 B).

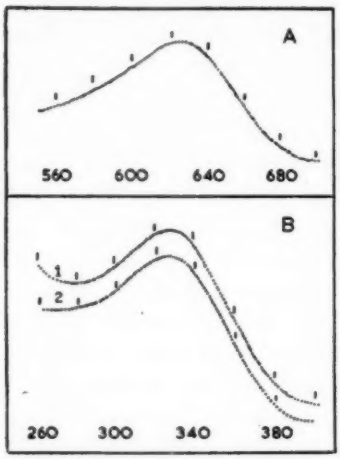

FIG. 5 A. Antimony trichloride reaction performed on a chloroform extract of the liver of *Coleonyx*. The peak at about 618 mμ is characteristic of vitamin A_1.

FIG. 5 B. The ultraviolet absorption spectrum of (*a*) a chloroform extract of the liver of *Coleonyx* (curve 1) and (*b*) a chloroform solution of crystalline vitamin A_1 (curve 2). All three curves of Fig. 5 were traced from original records made with a recording spectrophotometer.

DISCUSSION

A. The Absorption Spectrum of the Phyllurus Pigment.—Neither the absorption spectrum of the unbleached retinal extract (Fig. 1) nor the difference spectrum (Fig. 2) represents the true absorption spectrum of the visual pigment of this gecko. The former is distorted, especially in the region of shorter wave lengths, by the presence, in the extract, of yellow impurities. The latter is distorted also at shorter wave lengths by the occurrence of yellow products of beaching, although this effect is minimized in the NH_2OH experiment (Fig. 3). In spite of these distortions the absorption spectrum of a relatively pure extract and the NH_2OH difference spectrum, when plotted together on a comparable scale (Fig. 6), agree reasonably well down to about 520 mμ. Since these curves represent only one photosensitive component, it may be concluded that the

absorption curve of the pure pigment down to about 520 mμ is directly and accurately pictured by these results. Another useful procedure in the analysis of visual pigments is to apply the Dartnall nomogram (17). Using 524 mμ as the peak of the absorption spectrum the application of this nomogram yielded curve 3 (Fig. 6). This reconstructed curve agrees well with the experimental data down to about 500 mμ. Below this wave length the recon-

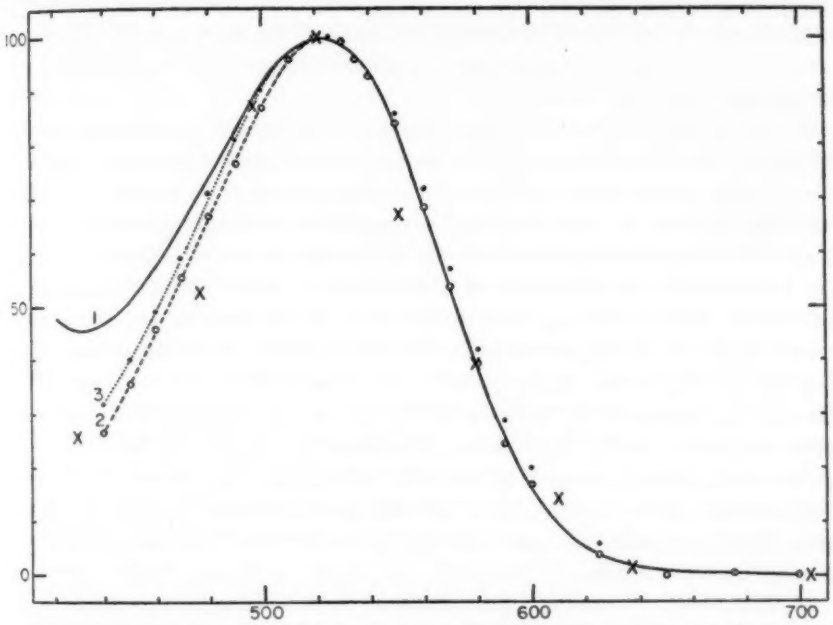

FIG. 6. Summary graphs which show the absorption spectrum of a relatively pure unbleached *Phyllurus* extract (curve 1), the NH_2OH difference spectrum (curve 2), and the reconstruction based on Dartnall's nomogram (curve 3). The points indicated as ×'s are from Denton's data on the visual sensitivity curve of *Gekko gekko* expressed on a quantum basis. All curves were scaled so that each maximum was at 100 per cent.

structed curve falls in between the other two curves but is closer to the difference spectrum. In the light of all this, two statements may be made about the *Phyllurus* pigment: (*a*) for all practical purposes the absorption spectrum is reasonably well established down to about 460 mμ by the reconstructed curve and (*b*) this chromoprotein is a typical broad banded visual pigment with an absorption spectrum which has the same form as the other visual chromoproteins fitted by the Dartnall nomogram (17). The only distinctive feature about it is its location at about 524 mμ which is unique for pigments in terrestrial animals.

B. Physiological Correlations.—It is now possible to correlate the justified portions of the absorption curves (Fig. 6) with Denton's spectral sensitivity data. Through the kindness of Dr. Denton, who made his data available to the writer, this correlation is shown in Fig. 6. The points marked with X's represent the sensitivity data. There is no reasonable doubt that the physiological and biochemical data are in accord. This agreement leads to two mutually related conclusions: (*a*) the visual sensitivity function of the gecko is satisfactorily explained in terms of the retinal pigment and (*b*) the *Phyllurus* chromoprotein is undoubtedly a visual pigment and is, therefore, of physiological interest.

C. The Visual Pigment of Other Geckos.—The general description of the *Phyllurus* pigment as given here is not unique for this species alone. The retinal extracts of six other species of nocturnal geckos were examined by the methods applied to the *Phyllurus* preparations. Though differences were noted in the spectral positions of the pigments from the different species, the two notable characteristics of the *Phyllurus* visual protein—location in the region, 518 to 528 mμ, and the presence of retinene$_1$ as a chromophore —also apply to the pigments from the other geckos. It is likely that these two characteristics also apply to other nocturnal geckos and to *Gekko gekko*, the species employed by Denton. It should be emphasized, however, that when applied to geckos as a whole, generalizations cannot yet be made. The geckos are a diverse group of animals and this is especially true from an ophthalmological point of view. Thus far only an insignificant number of genera have been examined and one important family, the Sphaerodactylidae, is missing from the results.

D. The Visual Pigment of Other Reptiles.—A few attempts have been made already to extract, and to identify, the visual pigments of reptiles (18, 19). These attempts have led to little success probably because of the presence of these proteins in such low concentrations in these cone-rich retinae. By a direct visual method Walls (20) noted the occurrence of a photolabile pigment in the retinae of several reptiles. He referred to it as visual purple. The finding of an unusual pigment in geckos raises the question whether a unique pigment, and not rhodopsin, is also a retinal constituent of other reptilian groups. Using the present methods, the successful extraction and identification of visual pigments have been accomplished in the case of two reptiles: the alligator (*Alligator mississippiensis*) and the Pacific rattlesnake (*Crotalus viridis helleri*).[3] In both cases rhodopsins were found. The difference spectrum of the alligator pigment is reproduced in Fig. 4 (curve 1). A similar difference spectrum was obtained as the result of bleaching the retinal extract of the rattlesnake. The gecko results need not be extrapolated to apply to other

[3] Dr. R. B. Cowles kindly provided the rattlesnakes and was most helpful in the risky experiment of handling these animals in the dark room.

reptiles. In the light of present information geckos appear to be unique among terrestrial animals in the possession of an atypical pigment system.

E. The Biological Significance of the Gecko Pigment.—A discussion of the significance of this unusual visual chromoprotein involves phylogenetic considerations. The basis for this statement is the transmutation theory of Walls (3, 4) which has received support from the studies of Underwood (5). According to this concept the ancestral gecko was a diurnal lizard with retinal cones. In the course of evolution a transmutation of cones to rods occurred in association with the development of the secretive, nocturnal habit. At some time during this evolution the high concentration of visual pigment in the outer segments is assumed to have developed. Underwood's interesting study of retinal cytology has brought to light a number of transition forms among living genera of geckos which support the transmutation theory in a convincing manner. Perhaps the most unexpected support for Walls' theory, in so far as geckos are concerned, is the finding by Crozier and Wolf (21) that the critical fusion frequency contour of the gecko, *Sphaerodactylus inaquae* Noble and Klingel, is similar to that obtained from *Pseudemys*, a turtle with a predominantly pure cone retina. Except for these few studies the transmutation theory has stood almost alone while overwhelming support has been marshalled in favor of the duplicity theory and no one, except for Walls' qualification about nomenclature, already referred to, seems to have questioned the view that the gecko visual pigment is anything but a typical rhodopsin. The present results, demonstrating, as they do, that this view is untenable, may be interpreted as supporting the transmutation theory. The so called rods of nocturnal geckos are characterized biochemically by a pigment of the retinene$_1$ class but one which is roughly intermediate in spectral position between the other two retinene$_1$ chromoproteins: the typical rod constituent, rhodopsin and the cone pigment, iodopsin. Is it possible that this arrangement is of evolutionary significance possibly representing substances which had their origin from a common ancestral retinene$_1$ pigment?

The data for the seven species of geckos from which retinal extracts were prepared suggest that the visual pigments are not spectroscopically identical in all these lizards. Minor, but significant, variations appear to exist in the position of the pigment from each of the seven species. It remains to be discovered whether a systematic, taxonomically significant variation occurs in the pigment system of geckos as a whole. Underwood (5) has recently proposed a classification of geckos into three families: (*a*) Eublepharidae, (*b*) Sphaerodactylidae, and (*c*) Gekkonidae. Transmutation is supposed to have occurred independently in these three gecko stocks. It will be of interest in future work to inquire whether the evolution of the visual pigment also occurred independently in these three families or whether the biochemical pattern was fixed in the common ancestral form. It would not be too surprising

to discover in future work, gecko pigments in several other spectral positions and all members of the retinene$_1$ system. Even typical rhodopsin may turn out to be present in some species. It will be of special interest to determine which, if any, visual pigment can be extracted from the retinae of those diurnal geckos whose retinal cells consist of cones presumably derived tertiarily from rods. The geckos appear to offer fruitful prospects for future physiological and biological queries.

F. The Matter of Nomenclature.—The discovery of a visual pigment at the position of porphyropsin yet containing retinene$_1$, raises the question of a proper nomenclature for this group of pigments. The classical system of describing them according to their color (rhodopsin, porphyropsin, iodopsin, or visual purple, visual violet) is obviously insufficiently informative in the case of the *Phyllurus* pigment which has the same color as the porphyropsins but which differs from them in the nature of its chromophore. The same objection can be made to Dartnall's system (22) of naming each pigment numerically according to the spectral location of the absorption maximum. There are several alternative methods which might be adopted including modification of the present systems by the addition of the subscript 1 or 2 according to whether retinene$_1$ or retinene$_2$ is obtainable from the products of bleaching. In view of the possibility of further discoveries in this field the present author is not yet prepared to offer suggestions for a definitive system of nomenclature even though it is clear that present systems are unsatisfactory in a number of respects.

SUMMARY

Retinal extracts of the Australian gecko, *Phyllurus milii* (White), have revealed the presence of a photosensitive pigment, unusual for terrestrial animals, because of its absorption maximum at 524 mμ. This pigment has an absorption spectrum which is identical in form with that of other visual chromoproteins. It is not a porphyropsin, for bleaching revealed the presence, not of retinene$_2$, but of retinene$_1$ as a chromophore. Photolabile pigments with characteristics similar to those of the *Phyllurus* visual pigment were also detected in retinal extracts of six other species of nocturnal geckos.

The presence of this retinal chromoprotein adequately accounts for the unusual visual sensitivity curve described by Denton for the nocturnal gecko. This pigment may have special biological significance in terms of the unique phylogenetic position of geckos as living representatives of nocturnal animals which retain some of the characteristics of their diurnal ancestors. The occurrence of this retinene$_1$ pigment, intermediate in spectral position between rhodopsin and iodopsin, is interpreted in support of the transmutation theory of Walls. The results and interpretation of this investigation point up the fact that, from a phylogenetic point of view, too great an emphasis on the

duplicity theory may serve to detract attention from the evolutionary history of the retina and the essential unitarianism of the visual cells.

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A NEW PHOTOSENSITIVE PIGMENT OF THE EURYHALINE TELEOST, GILLICHTHYS MIRABILIS

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(Received for publication, March 19, 1956)

INTRODUCTION

In a recent review (1955) of his work on the visual and other carotenoid pigments of the vertebrate retina Wald has taken the position that there are two known visual pigments, rhodopsin and porphyropsin, which appear, either singly or in combination, to be involved in scotopic vision. It has been demonstrated that rhodopsins (wave length of maximum absorption near 500 mμ) are usually present in those marine fishes and terrestrial vertebrates with rod-containing retinae (summary by Krause, 1951). The fresh water fishes (Wald, 1939 *b*), on the other hand, possess porphyropsins (λ_{max} near 522 mμ) as the chief visual pigment of scotopic vision. It should be mentioned, however, that Dartnall (1952 *a*), to cite only a single paper, obtained evidence that photosensitive pigments of the fresh water tench and pike absorb light maximally at 533 mμ. This apparent variation in the spectral position of the retinal pigments of fresh water fishes is probably not completely understood. The photosensitive retinal pigment of the carp was examined by Crescitelli and Dartnall (1954), who found a λ_{max} at 523 mμ, confirming for this fresh water species Wald's general statement on the nature and occurrence of porphyropsin.

The euryhaline and migratory teleost fishes have been a source of special interest. Wald (1941) showed that spectral absorption curves of retinal extracts of such fishes (*Anguilla*, *Fundulus*, *Oncorhynchus*, *Salmo*, and *Salvelinus*) were intermediate between rhodopsin and porphyropsin. Because he obtained evidence that mixtures of the vitamins A_1 and A_2 occur in the retinae of these fishes, Wald inferred that both rhodopsin and porphyropsin are present in their retinae, in such proportions as to give the summed spectral

* To Dr. Frederick Crescitelli, Department of Zoology, University of California, Los Angeles, are due my deepest thanks; for without his constant encouragement and instruction in technique and interpretation this work could not have been attempted or completed. Much of the equipment required for this study was made available by Dr. Crescitelli's grant from the Division of Research Grants and Fellowships, National Institutes of Health, United States Public Health Service.

absorption curves which he measured. There has been no direct analysis, however, of the visual pigments to test this plausible idea.

The investigation reported here was performed with retinal extracts of a euryhaline teleost, the longjaw goby or mudsucker, *Gillichthys mirabilis* Cooper. This species is a member of the family Gobiidae, a group of primarily marine fishes usually found in shallow coastal waters. Many of the species, however, are tolerant of reduced salt concentrations or may even be restricted to fresh water. *Gillichthys mirabilis* is common on tidal mudflats and salt marshes of the Pacific coast of North America from northern California to southern Baja California and in the Gulf of California. The mudsucker has a marked ability to endure for extended periods salt concentrations both above and below that of sea water (Weisel, 1948). The effect of varied dietary amounts of vitamin A on the quantity of visual pigment in the mudsucker retina was studied by Kampa (1953).

This paper describes experiments with retinal extracts of the euryhaline fish, *Gillichthys mirabilis*, and presents evidence that these extracts contain a single photosensitive pigment different from those of other animals so far examined by this method.

Materials and Methods

Adult mudsuckers (*Gillichthys mirabilis*, total length 10 to 15 cm.) were purchased from a local bait dealer. The fish were originally trapped in San Francisco Bay, California, and had been kept without feeding for an average period of 1 to 2 weeks in transport and in the dealer's holding tanks. The techniques used in this study have been described extensively elsewhere (Wald, 1938; Collins and Morton, 1950). All operations were performed in dim red light; 4 per cent potassium alum solution was used to harden the retinal tissue. Squirting alum solution between the retina and pigment epithelium with a hypodermic needle (Kampa, 1953) aided the dissection. The retinal tissue was centrifuged from the alum solution, washed twice in distilled water and twice in alkaline (pH 8.6) borate–KCl buffer, and extracted twice with 2 per cent aqueous digitonin solution in borate–KCl buffer (pH 8.4). The extracts were kept at 10°C. until used.

A variant of this technique, described by Saito (1938) and Collins, Love, and Morton (1952), normally yields preparations freer from contaminants. After washing, the retinal material was shaken with 40 per cent sucrose solution, in which fragments of the outer segments of the visual cells became suspended. Centrifugation permitted separation from the retinal residue; dilution with distilled water and recentrifugation brought down the outer segments. Both outer segments and retinal residue were extracted with digitonin and examined spectrophotometrically.

After centrifugation the retinal extracts were analyzed in a Beckman model DU spectrophotometer with photomultiplier attachment. As a blank, 2 per cent digitonin solution was used; the temperature was regulated at 20 ± 1°C. Optical density of the extracts was measured from 700 to 340 mμ at 10 mμ intervals. For bleaching a Bausch and Lomb grating monochromator with interference filters

placed in the exit light path provided light of the desired wave length. Both the monochromator exit slit width (3.3 mμ spectral dispersion per mm. width) and length of bleaching time were varied to control the amount of bleaching which oc-

TABLE I

Preparation and Spectral Characteristics of Retinal Extracts

Extracts are of *Gillichthys mirabilis* unless otherwise indicated.

Extract aliquot	No. of animals	Date of analysis	Extract age	Type of preparation	Density 700 mμ	Maximum density	Minimum density	Ratio	Maximum	Minimum
		1955	*days*						mμ	mμ
1A	20	July 1	4	Whole retina	0.032	0.356	0.260	0.73	507	430
1B	20	July 5	8	" "	0.006	0.236	0.161	0.68	507	430
1C	20	July 7	10	" "	0.007	0.308	0.213	0.69	506	430
2A	20	Aug. 2	14	Whole retina	0.013	0.273	0.204	0.75	503	430
2B	20	Dec. 6	140	" "	0.000	0.243	0.195	0.80	504	430
2C	20	Dec. 14	148	" "	0.002	0.195	0.155	0.80	504	430
3A	22	July 25	5	Outer segment	0.003	0.090	0.038	0.42	510	420
3B	22	July 27	7	" "	0.000	0.081	0.033	0.41	510	420
3C	22	Aug. 8	19	Residue	0.002	0.152	0.095	0.63	507	430
4A	40	Aug. 4	9	Outer segment	0.002	0.263	0.133	0.51	510	420
4B	40	Nov. 23	120	" "	0.002	0.240	0.155	0.65	508	440
4C	40	Nov. 26	123	" "	0.000	0.203	0.131	0.65	508	430
4D	40	Dec. 3	130	Residue	0.000	0.260	0.249	0.96	490	450
4E	40	Dec. 10	137	"	0.001	0.288	0.276	0.96	490	450
4F	40	Dec. 16	143	"	0.001	0.229	0.217	0.95	488	450
Rana catesbiana		Feb. 26, 1956	4	Outer segment	0.000	0.272	0.100	0.37	502	390
Cyprinus carpio		" " "	12	" "	0.002	0.198	0.160	0.81	515	450
Rana and Cyprinus		" 27, "	—	" "	0.015	0.231	0.196	0.85	503	450

curred at any wave length. Following exposure to light the extracts were replaced in the spectrophotometer and the absorption spectrum measured as before. In this way the effects on each extract of several bleaching wave lengths were studied successively.

RESULTS

1. Spectral Absorption Curves of Mudsucker Retinal Extracts.—Some of the properties of different unbleached aliquots of four extracts prepared from mudsucker retinae are listed in Table I. The absorption maxima which were

obtained were generally intermediate between the λ_{max} of rhodopsin and of porphyropsin. The range of the wave length of maximum absorption (λ_{max}) of the mudsucker extracts from 488 to 510 mμ indicates either that the λ_{max} is not an unbiased indicator of the wave length of maximum absorption of the mudsucker retinal pigment or that different pigments (or the same pigments in different proportions) were present in these extracts. Experiments

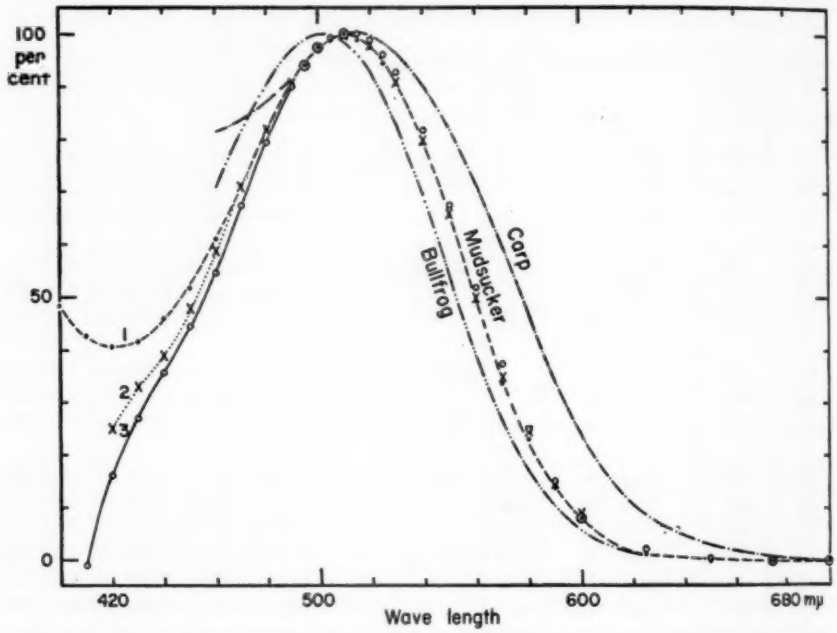

FIG. 1. Curve 1, absorption spectrum of a relatively pure retinal extract of the mudsucker (D_{min}/D_{max} = 0.42); curve 2, constructed from Dartnall's nomogram, assuming a maximum at 512 mμ; curve 3, hydroxylamine experiment, difference spectrum after exposure of mudsucker extract 4C to red light (λ = 606 mμ). Absorption spectra of retinal extracts of the bullfrog and carp are given for comparison.

in which the extracts were bleached with monochromatic light resolved this problem (see section 2 below). A comparison of the ratio of the optical densities of the preparations at their points of minimum and maximum light absorption (D_{min}/D_{max} ratio) and the λ_{max} of the extracts listed in Table I shows that the lower the ratio, the longer was the wave length of maximum absorption. Preparations with lower D_{min}/D_{max} ratios contained smaller proportions of violet-absorbing, photostable substances; their spectral absorption curves, therefore, better represent the actual spectral absorption of the photosensitive pigment(s) present.

The spectral absorption curve of the unbleached extract aliquot 3A, an outer segment preparation with the relatively low D_{min}/D_{max} ratio 0.42, is shown as curve 1 of Fig. 1. The experimental points from which this curve was constructed are plotted as percentages of the maximum (λ = 510 mμ). If a single photosensitive pigment were present in the mudsucker retinal extracts, this curve should fairly well represent its spectral absorption down to about 460 mμ. For comparison spectral absorption curves of bullfrog (*Rana catesbiana*) rhodopsin and carp (*Cyprinus carpio*) porphyropsin, obtained in this work, are also included in Fig. 1. These curves differ at all wave lengths from the spectral absorption of the mudsucker extract, and not merely at λ_{max}. For interpretation of the spectral absorption curves of the mudsucker extracts it was necessary to demonstrate the presence of photosensitive pigment in these extracts and to determine whether more than one component was present in this pigment.

2. Homogeneity of the Mudsucker Photosensitive Pigment.—That the retinal extracts which were examined contained light-sensitive pigments is shown in Table II, in which the optical density losses following exposure to light are recorded. To show whether one or more components were bleached in these experiments required an analysis using a technique of partial bleaching. This is the method employed by Crescitelli and Dartnall (1954) in their work on the carp. The retinal extract is exposed to monochromatic light and bleached in stages. If the change in optical density of the extract's spectral absorption (difference spectrum) is plotted after each exposure, a shift in the wave length of maximum change indicates the bleaching successively of more than one photosensitive component. A constant λ_{max} of the successive difference spectra means, on the other hand, that only a single, homogeneous pigment has been bleached. In the present work care was taken in the exposure of extracts to light of shorter wave lengths, reported to isomerize the products of bleaching (Hubbard and Wald, 1952; Hubbard, Gregerman, and Wald, 1953). The spectrophotometric analyses were performed with the retinal extracts at pH 8.4 to shift the absorption maximum of the products of bleaching as far as possible from the λ_{max} of the visual pigment. This minimized distortion of the difference spectrum due to the appearance of violet-absorbing products of bleaching.

The results of all bleaching experiments performed with aliquots of mudsucker extracts 1 through 4 are listed in Table II. Difference spectra were obtained as a result of exposure to light at a number of wave lengths from 660 to 390 mμ. The difference spectra are almost the same, the λ_{max} ranging from 512 to 517 mμ with an average near 514 mμ. After bleaching of this pigment with colored light was nearly complete, exposure to white light was sometimes used to determine whether any other previously unbleached component might still be present in the extracts. A difference spectrum charac-

TABLE II

Bleaching Experiments

Total change is the loss in optical density at 515 mμ due to all bleaches. When an extract was bleached several times, the results are tabulated with the first on top and the successive ones below.

Extract aliquot	Hydroxylamine	Total change	Bleaching wave length	Wave length of maximum difference	Maximum density loss
			mμ	mμ	
1A	No	0.320	660	517	0.089
			580	514	0.231
1B	No	0.215	660	513	0.092
			580	512	0.123
1C	(No)*	0.280	660	514	0.171
			580	514	0.109
2A	No	0.223	640	515	0.104
			606	515	0.119
2B	No	0.214	606	516	0.119
			640	514	0.055
			W‡	Isom.§	—
2C	Yes‖	0.171	390¶	512	0.147
			606	512	0.024
3A**	No	0.076	606	515	0.076
3B**	(No)	0.073	606	513	0.073
3C	No	0.121	640	513	0.056
			606	515	0.065
4A**	No	0.245	640	515	0.060
			640	Indefinite‡‡	0.040
			606	515	0.079
			606	512	0.067
4B**	No	0.198	640	515	0.060
			640	515	0.036
			606	515	0.050
			606	515	0.052
			W	Isom.	—
4C**	Yes	0.190	606	512	0.181
			W	—	—
4D	Yes	0.217	640	512	0.174
			606	512	0.037
			W	—	—
4E	No	0.233	640	515	0.074
			606	515	0.145
			W	Isom.	—
4F	Yes	0.176	390	512	0.051
			606	512	0.126

TABLE II—*Concluded*

Extract aliquot	Hydroxylamine	Total change	Bleaching wave length	Wave length of maximum difference	Maximum density loss
			mμ	mμ	
Rana and *Cyprinus***	No	0.170	640	525	0.043
			640	515	0.026
			606	510	0.033
			606	504	0.047
			W	Isom.	—

* Only 0.02 ml. of 0.1 M NH_2OH was added; this was too small a quantity to have any marked effect.

‡ W means that a tungsten filament bulb (100 watts) at a distance of 6 inches was used as the bleaching source.

§ Isomerization of the bleaching products resulted in a small change; negligible additional bleaching of pigment occurred. See Fig. 3.

‖ 0.1 ml. of 0.1 M NH_2OH was added to both extract and blank.

¶ No interference filter was available for the wave length 390 mμ.

** Outer segment preparations; others were extracts of the residues or of whole retinae (see Table I).

‡‡ The peak of this small difference was flat and not clearly marked.

teristic of the bleaching of a visual pigment was in no case obtained after such a terminal exposure to white light. Curve 5-6 of Fig. 3 B shows the typical result: a low, ill-defined, often more or less two-peaked change. This effect was also observed following a final white light bleach of an artificial mixture of bullfrog rhodopsin and carp porphyropsin (Fig. 4 B, curve 5-6). The use of hydroxylamine, which combines with retinene to form the oxime, largely prevents such effects (Hubbard and Wald, 1952; Wald and Brown, 1953). Curve H of Fig. 3 B shows the change due to terminal exposure to white light of another aliquot (4C) of the same extract, but one to which hydroxylamine had been added. The suppression in the hydroxylamine-treated aliquot of the white light effect makes it clear that no additional visual pigment had been bleached by the white light. Partial bleaching ef extract aliquots 2C and 4F (to which hydroxylamine had been added) with violet light of 390 mμ gave a difference spectrum maximum of 512 mμ. This figure was constant for the hydroxylamine difference spectrum and is also evidence for the lack of other light-sensitive pigments in the mudsucker extracts.

Graphic evidence for the homogeneity of the mudsucker photosensitive pigment is contained in Fig. 2, which presents averaged data for the difference spectra obtained after all bleaches (in extracts to which hydroxylamine was not added), plotted as percentages of the maxima. The broken line represents the grand mean of the difference spectra obtained after all 24 bleaches, and the points the means of the difference spectra obtained after exposure to each

selected wave length from 580 to 660 mμ. The averaged results of bleaches at these individual wave lengths are seen to match closely, especially at λ_{max} and in their longer wave length segments. This uniformity indicates that the photosensitive pigment present in these retinal extracts was a single, homogeneous pigment.

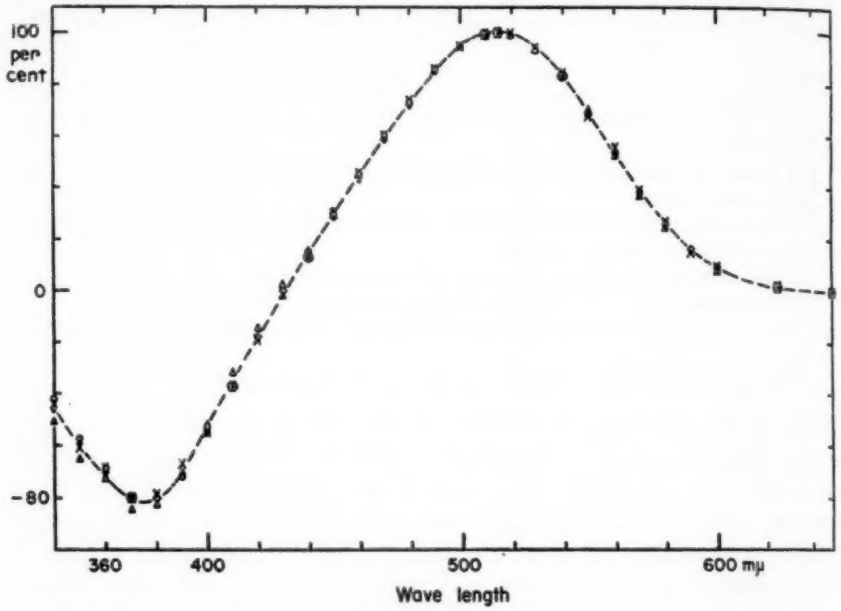

FIG. 2. Averaged difference spectra scaled with maxima set equal to 100 per cent. Broken line, grand average of all bleaches. ×'s, average of 3 bleaches at 660 mμ (maximum total change 0.330 optical density units); open circles, average of 8 bleaches at 640 mμ (change 0.483); points, average of 10 bleaches at 606 mμ (change 0.844); triangles, average of 3 bleaches at 580 mμ (change 0.462).

3. Comparison of the Mudsucker Extracts with a Mixture of Rhodopsin and Porphyropsin.—As a critical test of the homogeneity of the mudsucker retinal extract, two bleaches were performed at 640 mμ and two at 606 mμ, under conditions believed to differentiate clearly a mixture of rhodopsin and porphyropsin. The results of this experiment are given in Fig. 3. Fig. 3 A shows the actual spectral absorption curves measured initially and following each exposure to light and Fig. 3 B the successive difference spectra due to each exposure. These difference spectra are almost identical, showing a maximal change at 515 mμ; a final exposure to white light produced only the anomalous curve 5-6, already described. Curve H of Fig. 3 B shows how this white light effect was almost eliminated by the addition of hydroxylamine to another aliquot (4C) of the same solution, which had previously been bleached

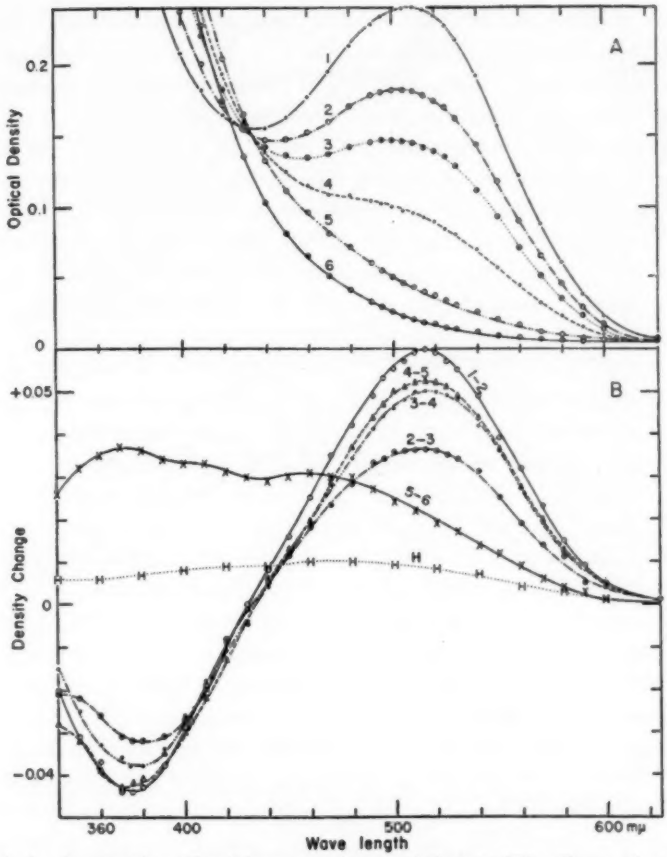

FIG. 3 A. A selective bleaching experiment (aliquot 4B). Curve 1, absorption spectrum of unbleached extract; curves 2 and 3, absorption spectra measured after successive 1 hour bleaches with 640 mμ light (monochromator exit 5 mm.); curves 4 and 5, absorption spectra measured after successive bleaches of 10 and 30 minutes, respectively, with 606 mμ light (exit 5 mm.); curve 6, absorption spectrum measured after 10 minute exposure to white light (100 watt tungsten bulb).

FIG. 3 B. Difference spectra of experiment with aliquot 4B. Positive changes (upward) indicate loss of density; negative changes (downward) indicate gain in density. Curves 1–2 and 2–3 are the results of the two 640 mμ bleaches; 3–4 and 4–5 are due to the exposures to 606 mμ light. Curve 5–6 is the final change due to exposure to white light. Curve H is the difference obtained with a final white light bleach in another aliquot (4C) of the same extract, but one to which hydroxylamine had been added.

by red light at 606 mμ (curve 3 of Fig. 1). This experiment suggests that only one photosensitive pigment was present in the extracts prepared.

To demonstrate the adequacy of the above method a control experiment was performed with an artificial mixture of bullfrog (*Rana catesbiana*) rho-

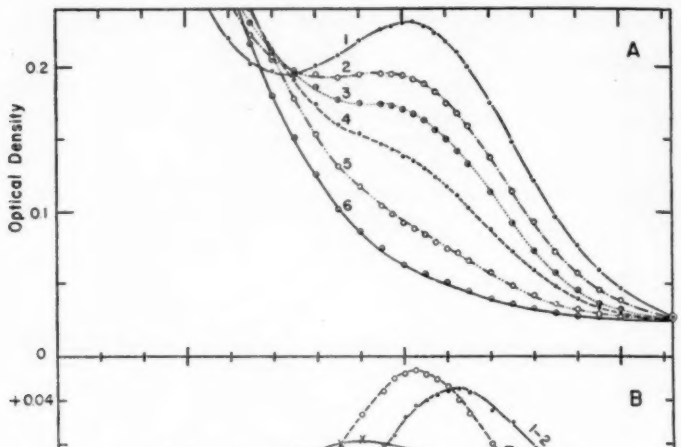

FIG. 4 A. A selective bleaching experiment with a mixture of bullfrog rhodopsin and carp porphyropsin. Curve 1, absorption spectrum of unbleached extract; curves 2 and 3, absorption spectra measured after successive 1 hour bleaches with 640 mμ light (monochromater exit 5 mm.); curves 4 and 5, absorption spectra measured after successive bleaches of 15 minutes and 1 hour, respectively, with 606 mμ light (exit 5 mm.); curve 6, absorption spectrum measured after 10 minute exposure to white light (100 watt tungsten bulb).

FIG. 4 B. Difference spectra of carp-bullfrog experiment. Curve 1–2 is the change due to the first exposure to 640 mμ light. Curve 4–5 is the result of the last 606 mμ bleach. Curve 5–6 is the final change due to exposure to white light. Omitted for the sake of clarity are curves 2–3 (second 640 mμ bleach) and 3–4 (first 606 mμ bleach); the characteristics of these difference spectra are listed in Table II. Both were intermediate between curves 1–2 and 4–5.

dopsin (λ_{max} 500 to 502 mμ) and carp (*Cyprinus carpio*) porphyropsin (λ_{max} 523 mμ). Both of these pigments are well known and have been adequately studied (Wald, 1938; Crescitelli and Dartnall, 1954). Outer segment extracts of two adult bullfrogs and of three carp were prepared separately in the man-

ner described previously. The absorption spectra of the bullfrog and carp extracts used are given in Fig. 1. The extracts were mixed so as to give the summed spectral absorption curve 1 of Fig. 4 A. This curve is similar to curve 1 of Fig. 3 A, the spectral absorption curve of mudsucker extract aliquot 4B. Again the series of two bleaches at 640 mμ and two at 606 mμ was completed; the only difference between the bleaching conditions of the two experiments was that somewhat longer bleaches at 606 mμ were given the bullfrog-carp mixture. As one would expect, rhodopsin is less sensitive than the mudsucker pigment to light of this wave length and requires longer exposure to be bleached proportionately. In Fig. 4 B the difference spectra obtained after the first exposure to 640 mμ light (curve 1-2) and after the last exposure to 606 mμ light (curve 4-5) are plotted. The shift from a porphyropsin (λ_{max} = 525 mμ) to a rhodopsin (λ_{max} = 504 mμ) difference spectrum is clear. The intermediate difference spectra would make the figure confusing and were left out for this reason. The difference spectrum 2-3 (second bleach at 640 mμ), however, was maximal at 515 mμ (see Table II). The curve 3-4 (first bleach at 606 mμ) was also intermediate (λ_{max} = 510 mμ) between the first and last bleaches. There was, therefore, a gradual shift in the difference spectra during the course of bleaching; the intermediate curves are due to partial bleaching of both components. In this way a mixture of two photosensitive pigments with absorption maxima only 20 mμ apart was shown to be distinguishable by the method of partial bleaching. This assures that the evident homogeneity of the mudsucker light-sensitive pigment is real and not the product of inadequate analytic methods. It should also serve as caution that the spectral absorption curve of a given retinal extract may represent a number of possible combinations of component pigments. An interpretation attempted without adequate demonstration of the constituent nature of such a total curve may be subject to error.

4. The Hydroxylamine Difference Spectrum of Mudsucker Retinal Extracts.—An attempt to locate more prediscely the spectral absorption maximum of the mudsucker photosensitive pigment was made with the use of hydroxylamine. Hubbard and Wald (1952) stated that hydroxylamine combines with retinene to form the oxime. The absorption maximum of this oxime is shifted to shorter wave lengths than the λ_{max} of the indicator yellow. The use of hydroxylamine, therefore, results in a difference spectrum which is freer from distortion due to the absorption of light by the products of bleaching. This difference spectrum should, in fact, very closely approximate the true absorption spectrum of the photosensitive pigment to wave lengths as short as about 460 mμ. Curve 3 of Fig. 1 is such a hydroxylamine difference spectrum and agrees with the absorption spectrum of the purest retinal extract down to about 460 mμ. The maxima of the hydroxylamine difference spectra of aliquots 2C, 4C, 4D, and 4F were all at 512 mμ.

5. Spectrophotometric Analysis of Mudsucker Retinal Carotenoids.—Extrac-

tion and analysis of the retinal carotenoids were performed with the methods described extensively by Wald (1939 *a*, 1939 *b*). On June 13, 1956, 40 adult mudsuckers were obtained from a local bait dealer, and the right and left retinae of the dark-adapted fish were collected separately. The left retinae were extracted for 20 minutes in the dark with petroleum ether (b.p. 66-

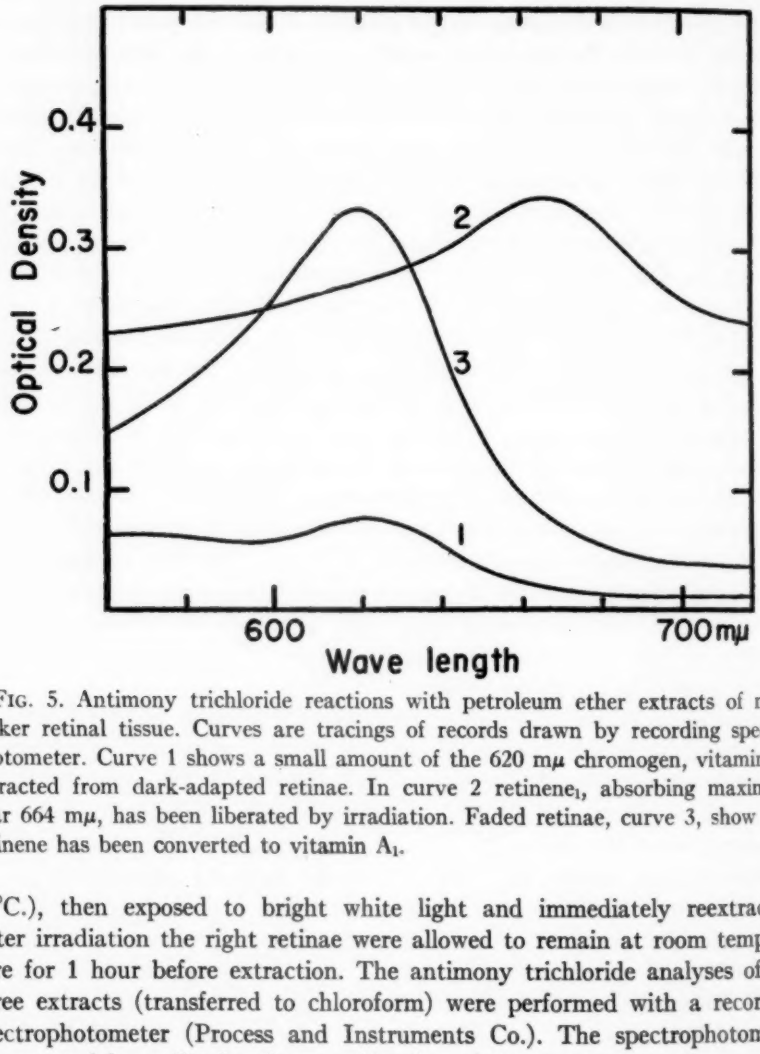

FIG. 5. Antimony trichloride reactions with petroleum ether extracts of mudsucker retinal tissue. Curves are tracings of records drawn by recording spectrophotometer. Curve 1 shows a small amount of the 620 mμ chromogen, vitamin A_1, extracted from dark-adapted retinae. In curve 2 retinene$_1$, absorbing maximally near 664 mμ, has been liberated by irradiation. Faded retinae, curve 3, show that retinene has been converted to vitamin A_1.

75°C.), then exposed to bright white light and immediately reextracted. After irradiation the right retinae were allowed to remain at room temperature for 1 hour before extraction. The antimony trichloride analyses of the three extracts (transferred to chloroform) were performed with a recording spectrophotometer (Process and Instruments Co.). The spectrophotometer was started immediately after combination of the reactants; each curve required about 2 minutes to record. Fig. 5 shows the results of these tests; a small amount of vitamin A_1 (λ_{max} 620 mμ) was obtained by preextraction in

the dark (curve 1). After exposure to white light (curve 2) a larger amount of retinene$_1$, absorbing maximally near 664 mμ, was recovered. From the other group of retinae which had been allowed to stand after exposure to light, vitamin A_1 was obtained (curve 3). After 30 minutes the absorption bands of all extracts almost entirely disappeared. No evidence of vitamin A_2 or retinene$_2$ was found. The data do suggest that the mudsucker photosensitive pigment is a member of the family of retinene$_1$ visual pigments, which include classical rhodopsins and iodopsin.

DISCUSSION

1. Absorption Maximum of the Mudsucker Photosensitive Pigment.—The location of the absorption maximum of the mudsucker photosensitive pigment may be estimated by considering the data together. The λ_{max} is certainly at a somewhat longer wave length than the absorption maximum (510 mμ) of the purest extract. As already stated, Fig. 1 combines such a curve with that of the hydroxylamine difference spectrum. The latter is a closer approach to the true pigment absorption spectrum than are the difference spectra (λ_{max} = 514 to 515 mμ) without hydroxylamine, which are distorted by the color of the violet-absorbing products of bleaching. Assuming 512 mμ to be the actual pigment maximum, a curve was constructed using Dartnall's nomogram (1953). This is curve 2 of Fig. 1. The nomogram is based upon the similarity in shape of known visual pigment absorption curves when frequency rather than wave length is plotted on the abscissa; the entire curve is merely translated according to the pigment's λ_{max}. The agreement among the three curves of Fig. 1 down to a wave length of 460 mμ is very good.

An attempt was made to correlate the spectral absorption maxima of the mudsucker extracts and their relative purity (indicated by the $D_{min.}/D_{max}$ ratio) in the manner employed by Crescitelli and Dartnall in their work (1954) on carp porphyropsin (λ_{max} = 523 mμ). In Fig. 6 is the same curve, which was the curve of best fit for the carp data, as in their Fig. 8; but the wave length scale is shifted as appropriate for a pigment maximum of 512 mμ. The open circles represent the absorption maxima of the extracts tested; they fit this line approximately (perhaps a slightly better fit would be obtained by drawing the line appropriate for a pigment at 511.5 mμ). The closed circles represent the maxima of the difference spectra without hydroxylamine and are scattered about the vertical line which is the average for the carp data also translated for a pigment with λ_{max} at 512 mμ. The half-filled circles are the λ_{max} of hydroxylamine difference spectra, and all fall at 512 mμ. Combination of all lines of evidence leads to the condlusion that the photosensitive pigment of the mudsucker has its absorption maximum at 512 $\pm$ 1 mμ and may, therefore, be named a retinene$_1$ pigment 512 of *Gillichthys mirabilis*.

The further note may be added here that a retinal extract prepared from

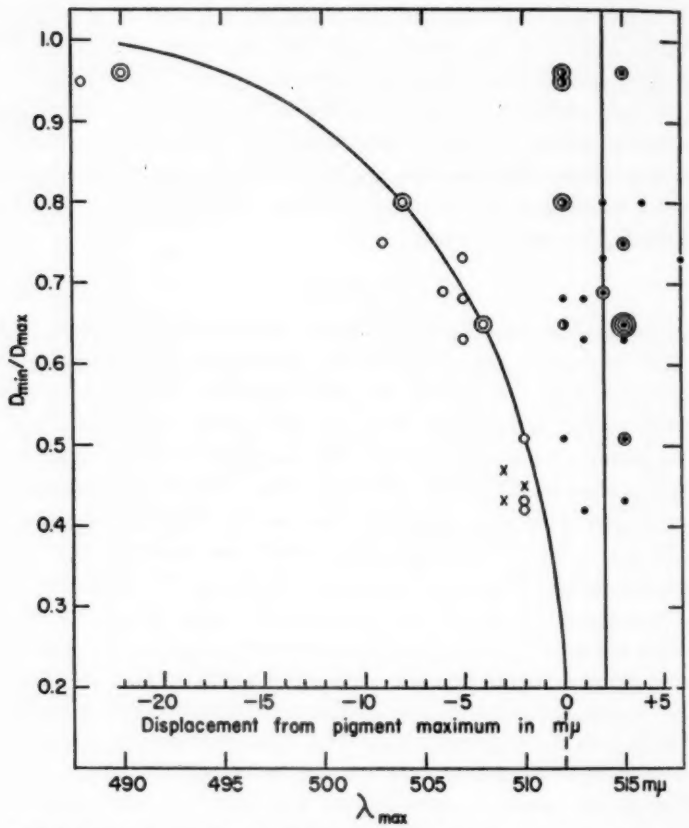

FIG. 6. Relation between the "purity" (D_{min}/D_{max} ratio) of retinal extracts and their λ_{max}. Solid line is curve of best fit for carp porphyropsin (Crescitelli and Dartnall, 1954), translated so λ_{max} = 512 mμ. Open circles are measured spectral absorption maxima. Vertical line is the average value of λ_{max} of the carp difference spectra, also translated as appropriate for a photosensitive pigment with its λ_{max} at 512 mμ. Filled circles are difference spectrum maxima obtained with each aliquot. Half-filled circles are hydroxylamine difference spectrum maxima. ×'s are three extract absorption maxima taken from Kampa's work (1953) on the mudsucker and plotted here for comparison.

members of another population of mudsuckers appeared to contain the same photosensitive pigment as those from San Francisco Bay. The mudsuckers used for this preliminary extract were collected[1] on May 14 and 15, 1955, at Fish Springs, Salton Sea, Riverside County, California, and were members

[1] Collected and kindly donated for this work by Mr. George W. Barlow, Department of Zoology, University of California, Los Angeles.

of a population introduced there. This first attempt at extraction unfortunately gave a very impure preparation, which could be studied only by the difference spectra obtained after exposure to colored light (λ = 424 to 660 mμ). The data are not tabulated, but the difference spectra were all maximal between 512 and 517 mμ and were consistent with the other results.

2. Comparison with Previous Studies of Photosensitive Pigments of Gillichthys and of Other Species.—The retina and certain visual functions of *Gillichthys mirabilis* have been studied before by Kampa (1953). Her histological examination showed that the mudsucker retina is duplex; in addition she obtained curves of critical fusion frequency typical of animals with a duplex retina. She also determined the effect of varied dietary amounts of vitamin A upon the weight of the retina and concentration of retinal pigment. She did not recognize that this pigment was different, however, but believed it to be rhodopsin. Dr. Kampa (Mrs. Brian Boden) graciously allowed her original results to be compared with those of the present experiments. In Fig. 6 the absorption maxima of three of her retinal extracts are related to their D_{min}/D_{max} ratios. They suggest a pigment maximum at a wave length of 510 or 511 mμ, not greatly different from the conclusion drawn in the present work.

Another light-sensitive pigment at about the same position (510 mμ) as the mudsucker pigment has been described in a short paper by Dartnall (1952 *b*) from the fresh water bleak (*Alburnus lucidus*); retinal extracts of this fish contained a small amount of a pigment absorbing maximally at 510 mμ in addition to larger quantities of another photosensitive pigment (λ_{max} = 533 mμ). It seems desirable at the present time to distinguish between the trace pigment of the bleak at 510 mμ and the photosensitive pigment of the mudsucker.

The mudsucker photosensitive pigment has not been directly shown to be a visual pigment. Spectral sensitivity of this species has not been determined. The concentrations of pigment obtained in the extracts, shape of the spectral absorption curve, and analysis of the retinal carotenoids, however, all indirectly support the possibility that the pigment is a visual pigment of the rods, quite probably functioning in scotopic vision.

The mudsucker photosensitive pigment is related to rhodopsins only to the extent that it is one of a family of pigments from which retinene$_1$ may be recovered. Whether the shift of the absorption maximum from 500 to 512 mμ is due entirely to difference in the protein part of the pigment molecule or partly to some isomeric rearrangement of the carotenoid groups might be difficult at present to determine. This work on the mudsucker in no way prejudices the possible correctness for other euryhaline fishes of Wald's inference (1941) that a mixture of rhodopsin and porphyropsin combines to give an intermediate visual pigment absorption maximum. The biological

significance of a visual pigment occurring in a euryhaline fish, and absorbing maximally between the peaks of rhodopsin and porphyropsin, is not understood.

SUMMARY

Retinal extracts have been prepared from dark-adapted mudsuckers by treatment of retinal tissue or of isolated outer segments of the visual cells with digitonin solution. The extracts were examined spectrophotometrically and found to absorb light maximally between the wave lengths of 488 and 510 mμ, depending on the proportion of yellow impurities and light-sensitive pigment present. This photosensitive pigment was shown to be homogeneous by partial bleaching of the extracts with monochromatic light of various wave lengths from 390 to 660 mμ. The mudsucker pigment was specifically demonstrated not to be a mixture of rhodopsin and porphyropsin; the adequacy of the method used to analyze such mixtures was shown by performing a control experiment with an artificial mixture of bullfrog rhodopsin and carp porphyropsin.

Comparison of the hydroxylamine difference spectrum and of the absorption maximum of the purest retinal extract located the mudsucker photosensitive pigment maximum at 512 ± 1 mμ. Extraction of retinal tissue with a fat solvent after exposure to white light gave a preparation which after the addition of antimony chloride reagent developed the absorption band maximal near 664 mμ, which is characteristic of retinene$_1$. If an hour intervened between exposure of the retinal tissue to light and extraction of the carotenoid, the antimony trichloride test gave a color band maximal at 620 mμ, characteristic of vitamin A_1. No evidence of retinene$_2$ or vitamin A_2 was obtained. The euryhaline mudsucker has, therefore, a photosensitive retinal pigment with an absorption maximum halfway between the peaks of rhodopsins and of porphyropsins and belonging to the retinene$_1$ system characteristic of rhodopsins. The pigment is therefore named a retinene$_1$ pigment 512 of the mudsucker, *Gillichthys mirabilis*. It is uncertain whether this type of photosensitive pigment will be found in other euryhaline fishes.

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THE EXCITABLE PROPERTIES OF THREE TYPES OF MOTOR AXONS

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(Received for publication, June 6, 1956)

In previous descriptions of the three types of motor axons found in the walking legs of crustacea, it was suggested that these fibers have quite dissimilar excitable characteristics (Wright and Adelman, 1954; Wright and Coleman, 1954). In the experimental work reported here, there were many distinct differences in procedures with respect to the earlier work. Also, the data obtained by Wright and Adelman were from the crayfish, *Cambarus bartoni*. The present work on the normal excitable properties of lobster axons was undertaken to seek further evidence for the aforementioned contention, and to extend our information about the excitable process itself.

Methods

Opener, slow closer, and fast closer motor fibers from the limbs of the lobster, *Homarus americanus*, were studied by the method of Wright, Coleman, and Adelman (1955). Stimulation and recording techniques were as previously described (Adelman, 1956 *a*). Essentially, all responses were initiated and recorded at an oil-saline interface, and may be considered as derived from axons immersed in physiological solution. Cole's *Homarus* solution was used throughout the study (W. Cole, 1941).

RESULTS

(*a*) *Responses to Constant Current Stimulation.*—One of the most striking properties of these axons was their ability to respond with more than one action potential when stimulated with long duration currents of slightly greater than rheobasic strength. This property of repetitive discharge was present in all three types of fibers investigated. Occasionally, a non-repetitive fast closer axon was prepared, but this was more often the exception than the rule. For the most part, a given voltage determined a sequence of impulses for a given duration of current. In fast closer fibers, this total number of elicited spikes was usually less than the total number of spikes elicited by

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comparable voltages (in terms of rheobase multiples) from either openers or slow closers.

Upon constant current stimulation, as the strength was increased beyond the voltage which gave a long train of spikes, a number of the spikes began to "drop out" of the sequence. This phenomenon usually resulted in low

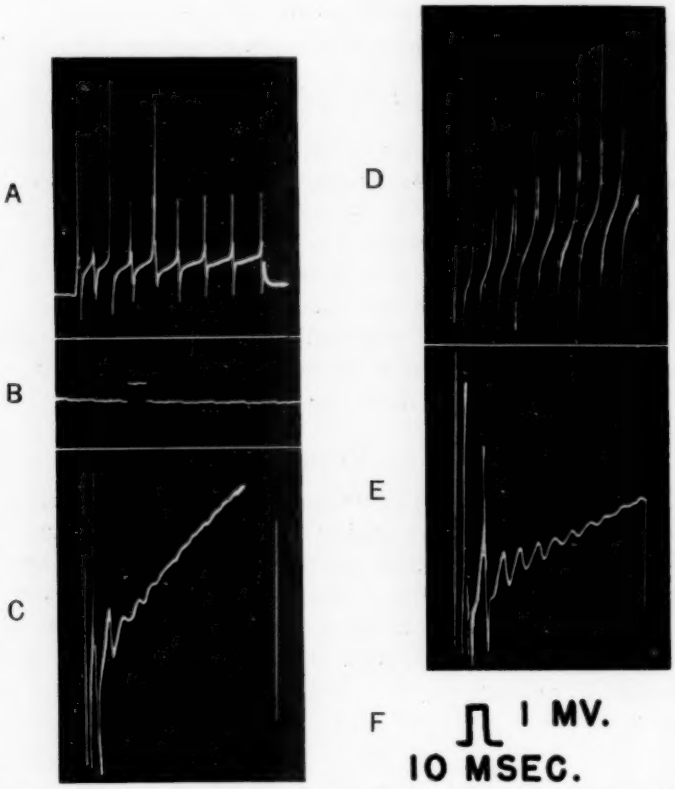

FIG. 1. Repetitive responses obtained from a twin fiber preparation (fast and slow closers). Cathodal depression. (See text.)

amplitude potentials appearing in the places where the spikes had been. This cathodal depression of the repetitive responses of a twin fiber preparation (fast and slow closers) is shown in Fig. 1. In *A* the action potentials of these two fibers elicited when they were stimulated simultaneously by an 100 msec. constant current are shown at relatively low amplifier gain. The stimulating voltage was about two times the rheobasic voltage for the slow closer. *B* represents the amplifier response to a 10 mv., 10 msec. signal at the gain used in *A*. Records *C*, *D*, and *E* show responses at high gain. Calibration of

the amplifier response at this high gain may be seen in *F*. *C* was obtained by stimulating at 6 times, *D* at 3.6 times, and *E* at 4.3 times the rheobasic voltage for the slow closer fiber. In records *C* and *E*, extreme cathodal depression

TABLE I

Rheobase Values of Single Lobster Motor Axons in Physiological Solution

Openers		Slow closers		Fast closers	
Fiber No.	Rheobase	Fiber No.	Rheobase	Fiber No.	Rheobase
	mv.		mv.		mv.
1	36	14	220	41	192
2	42	15	146	42	360
3	85	16	63	43	125
4	33	17	217	44	83
5	44	18	62	45	125
6	45	19	102	46	70
7	148	20	134	47	90
8	68	21	155	48	130
9	74	22	130	49	111
10	155	23	100	50	49
11	120	24	138	51	57
12	53	25	43	52	300
13	38	26	127	53	80
		27	26	54	160
		28	45	55	340
		29	150	56	48
		30	103	57	90
		31	65	58	250
		32	45	59	103
		33	70	60	75
		34	60	61	100
		35	50	62	70
		36	133	63	70
		37	55	64	215
		38	43	65	130
		39	58		
		40	175		
Means	72		101		137
Standard errors of mean	±12		±11		±18

may be seen with damped low amplitude potential oscillations. The repetitive response changed directly into the depressed state as the current was increased past a critical value.

Another aspect of constant current stimulation was the general loss of repetitive firing with fiber aging. This was apparently due to an increase of accomodation with time after preparation (Wright and Adelman, 1954).

Again, an order was seen in this phenomenon with respect to fiber type. Fast closers maintained their ability to respond repetitively to constant currents for shorter times after preparation than either slow closers or openers.

(*b*) *Rheobase Values.*—In determining the rheobase values of the individual motor axons, the oil-saline interface level (cathode) was set at approximately the same distance (0.5 cm.) from the anode on each fiber. The individual rheobase values so determined are given in Table I. The mean values were 72 mv. for the opener axons, 101 mv. for the slow closer axons, and 137 mv. for the fast closer axons. The appropriate standard errors of the means are given

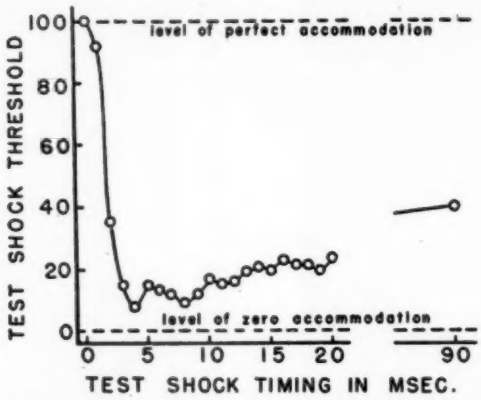

FIG. 2. Typical experimental curve for the variation in threshold as induced by subthreshold constant current conditioning. The "test shock" method. Ordinate, the test shock threshold in per cent of the unconditioned threshold. Abscissa; time interval between the "make" of the conditioning shock and the appearance of the test shock. Subthreshold conditioning was 90 per cent of the rheobasic voltage. See text.

in the table. There appears to be a small but significant difference among the rheobase values of the three fiber types. The order of these mean rheobase values was the same among the fiber types as the order of repetitive firing ability. The axonal type which fired the most repetitively also possessed the lowest rheobase values.

The considerable variation among the individual rheobase values of any one fiber type may have been due to varying stimulating electrode contact with the fibers from preparation to preparation, or to slight variations in the interelectrode distance. However, it would be strange, indeed, if there were no variance among fibers of a particular type.

(*c*) *Threshold Changes on the Application of Subthreshold Constant Currents.*—Measurements were made of the excitatory states as revealed by the variation in threshold to a very short duration "test shock" (Erlanger and Blair,

1931) when a cathodal subthreshold constant current was applied to these single fibers. A typical curve obtained from a slow closer fiber is seen in Fig. 2. Details of the method employed in obtaining and plotting such data are

TABLE II

Utilization Time Estimates (t_u) Determined from Curves Obtained by the "Test Shock" Method (See Text)

I. Twin fiber preparations (fast and slow closers)

Slow closers		Fast closers		Difference in t_u
R	t_u	R	t_u	
mv.	*msec.*	*mv.*	*msec.*	*per cent*
48	3.5	78	2.5	28.5
70	6.5	100	2.5	61.5
63	2.5	75	1.0	60.0
38	4.0	65	2.5	37.5
Means....55	4.1	80	2.1	46.9

II. All fiber types

Openers	Slow closers	Fast closers
t_u	t_u	t_u
msec.	*msec.*	*msec.*
4.5	6.5	3.5
10.0	2.5	2.5
9.0	6.5	2.5
24.0	4.5	4.5
10.0	6.5	1.0
	3.5	2.5
	6.5	1.0
	4.5	
	7.0	
	4.0	
	12.0	
	10.0	
Means.....11.5	6.2	2.5

R = rheobase, t_u = utilization time of the excitatory state.

given by Wright, Coleman, and Adelman (1955). In all fibers there was a tendency for the test shock threshold pattern to oscillate slightly with subthreshold conditioning. This mild oscillation may have been due to experimental error in determining the points. However, on repeated determinations the same oscillatory patterns could be seen again and again, and were always greater in magnitude than the expected errors inherent in the method. Also

this oscillation damped out with increasing time after the "make" of the constant current. Such oscillations in threshold, obtained by this method, have been seen by LeFevre (1950) in the squid giant axon, and have been recently noticed by Wright (1955) in lobster axons.

If the variations in threshold at the cathode seen on application of a subthreshold conditioning shock are related to the time course of the excitatory state as predicted by the two factor theories (Monnier, 1934) then the minimum threshold should represent the maximum of the excitatory disturbance, and thus occur at a time after the "make" of the conditioning shock equal to the utilization time, t_u. In determining t_u, the time corresponding to the minimum test shock threshold was taken as the utilization time. Table II

TABLE III

Time Constant of Accommodation (τ_2) Values for Single Lobster Motor Axons

Openers	Slow closers	Fast closers
75	100	14
89	85	90
100	90	80
85	83	14
106	98	77
85	60	83
Means.....90	86	60

shows the utilization times of the three types of fibers as determined by this method. A comparison between such values obtained from fast and slow closer axons prepared simultaneously is shown in part I of the table. About a 47 per cent difference between the utilization times of these fibers was found and is indicated in the table. In part II of Table II the individual t_u values of all fibers so estimated are shown. The mean values were 11.5 msec. for openers, 6.2 for slow closers, and 2.5 for fast closers. That these fibers are maintained at different levels of excitability is again evident from these data.

(*d*) *Excitability Constants.*—In Table III may be seen time constants of accommodation derived from data taken from the three types of fibers under identical conditions. Using exponentially rising currents (Solandt, 1936), estimates were obtained for Hill's λ or Monnier's τ_2 from the inverse slope of a plot of the threshold in rheobase units *vs.* the time constant of rise of the stimulating current. From the mean values obtained for τ_2 and the utilization time, t_u (obtained previously by the "test shock" method), the excitatory states were calculated for the different fibers from the general two factor excitability equations (Monnier, 1934). The relation between the utilization

time and the time constants of the excitatory state is:

$$t_u = \frac{\ln\left(\frac{\tau_2}{\tau_1}\right)}{\frac{1}{\tau_1} - \frac{1}{\tau_2}}$$

The calculated τ_1 values were 3.4 msec. for openers, 1.5 msec. for slow closers, and 0.5 msec. for fast closers. The observed τ_2 values were 90 msec. for open-

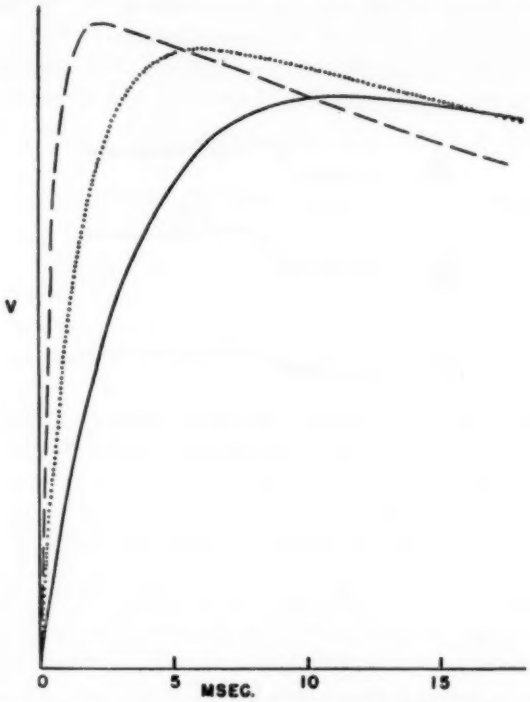

FIG. 3. Theoretical curves for the excitatory states of three types of motor axons. Constant current stimulation. See text. Solid line, opener; dotted line, slow closer; dashed line, fast closer.

ers, 86 msec. for slow closers, and 60 msec. for fast closers. LeFevre (1950) claimed that when $\tau_2/\tau_1 = 20$, there was about a 17 per cent overestimate of the real value of τ_2 when the estimates were obtained by the Solandt method. This overestimate follows directly from the assumption made by Hill (1936) in deriving the mathematical basis for the Solandt method, namely, that τ_2 must be many times greater than τ_1 in order to determine its value. Since almost all the τ_2/τ_1 ratios were relatively large, overestimates of τ_2 probably were small (somewhat less than 17 per cent).

It became apparent that it would be possible to plot the development of the excitatory state (V) based on the estimates for the time constants using the general equation for the excitatory state (Monnier, 1934):

$$V = Ri(e^{-t/\tau_2} - e^{-t/\tau_1}).$$

This equation predicts that for any applied constant voltage (Ri) the excitatory state (V) will rise to a maximum and then decline toward zero. The time course of the rise and fall of the excitatory state will be determined by the values of τ_1 and τ_2, which are time constants set by the tissue. By setting Ri equal to unity, $\tau_1 = 3.4$; $\tau_2 = 90$ for openers, $\tau_1 = 1.5$; $\tau_2 = 86$ for slow closers, and $\tau_1 = 0.5$; $\tau_2 = 60$ for fast closers, V was calculated for various arbitrary values of t (msec.). In Fig. 3 the V values so obtained are plotted

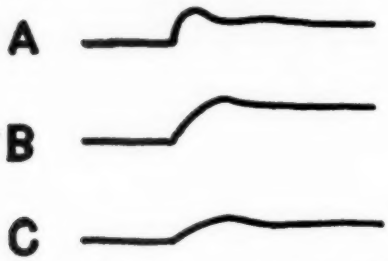

FIG. 4. Typical "local" or "initial" potentials obtained with just subthreshold constant current stimulation. Tracings of original records. See text. *A*, fast closer. *B*, slow closer. *C*, opener.

against time and represent the theoretical excitatory states for the three fiber types.

(*e*) *Local Responses.*—In a few cases, by lowering the oil-saline interface to the ground electrode, responses could be recorded directly from the stimulating cathode. Opener fibers normally showed a slowly developing local response, with its peak or maximum developing from 5 to 15 msec. from the beginning of an applied subthreshold constant current. There was a general similarity between the time course of the local response and the development of the excitatory state. With subthreshold constant current stimultation, the local response occasionally showed mild damped oscillations following the initial development. Fig. 4 is representative of such local potential recordings. *A* represents the local potential of a typical fast closer fiber; *B* represents the local potential of a typical slow closer fiber, and *C* represents the local response of a typical opener fiber. The time intervals between the "make" of the current and the peak of the local potential were 2.5 msec. for the fast closer, 5 msec. for the slow closer, and 6 msec. for the opener. These values are similar to the values for the excitatory state utilization times for the three

fiber types as determined by the "test shock" method. Consequently, there seemed to be a rather close parallelism between the development of the excitatory state and the development of the local potential.

DISCUSSION

From the foregoing results it seems apparent that the three anatomically different types of lobster motor nerve fibers possess different excitabilities. It is suggested that the more lengthy repetitive discharges seen in openers are a result of longer lasting and less accommodative local excitatory states. Prosser and Chambers (1938) found that the chronaxies of uncut giant fibers of the stellar nerves of the squid, stimulated *in situ*, were less than the chronaxies of uncut fin nerves of the same animal. Thus Prosser and Chambers were able to demonstrate a difference in excitability for anatomically different invertebrate nerve fibers.

Hodgkin (1948) found that *Carcinus* axons could be classified into three groups with respect to their ability to fire repetitive responses to constant current stimuli. Hodgkin found that the group of fibers which showed the widest range of frequency of firing, upon D.C. stimulation, also showed the longest utilization times, indicating either an absence of accommodation or an extremely slow primary excitable process. By the same token, the axons with the highest rheobases and the lowest safety factors, were shown to be either non-repetitive or only mildly repetitive upon constant current stimulation. Hodgkin also implied that there was a marked resemblance between the potential changes which precede the first spike in a repetitive sequence and those which precede any other spike in that sequence. Consequently, Hodgkin concluded that the frequency of repetitive firing with mild cathodal constant stimuli was determined by the response or utilization time rather than by the refractory period in the class of axons firing at slow frequencies (class I). However, in class II fibers a pronounced supernormal phase was observed. These axons tended to show little variation in frequency of repetitive firing upon variation in stimulus strength (in contradistinction to class I axons). Hodgkin concluded that the frequency of firing was determined by the form of the recovery curve and the rate of growth of the local response (interaction between supernormality and utilization time). However, he cautioned that it would be impossible to predict the frequency of the repetitive discharge from the recovery curve alone.

It would seem that the three classes of axons described by Hodgkin were not truly anatomical classes discovered by physiological means. Since no attempt was made by Hodgkin to present an anatomical-physiological relationship for his axons, close comparison between the fiber types used in this work and those used by Hodgkin becomes difficult. For the most part, opener fibers and slow closer fibers were similar to class I *Carcinus* axons, while fast

closer fibers more closely resembled class III *Carcinus* fibers. Since the supernormal phase was not estimated in this work, and since none of the lobster fibers showed such narrow limits of repetitive frequencies as class II *Carcinus* axons, comparisons of the lobster fibers with these fibers could not be made.

Easton (1952) showed in bundles of nerve fibers prepared from the leg nerve of *Cancer magister*, that different strength-duration curves corresponded to different sized action potentials produced by fibers in these bundles. The larger recorded spikes had shorter latencies, higher rheobases, and shorter chronaxies than the smaller recorded spikes. Whether Easton was dealing with fast and slow closer fibers or some other unknown fibers remains obscure, particularly since Easton made no attempt to discover which fibers in his prepared bundles were responsible for the different spikes. However, Easton guessed that the larger spikes, rather than the smaller spikes, belonged to larger diameter fibers.

Wright and Coleman (1954) and Wright and Adelman (1954) demonstrated similar differences in excitability among fast closers, slow closers, and openers in lobsters and crayfish as has been shown in this report. Wright, Coleman, and Adelman (1955) found that when these same fiber types were stimulated in sea water with a distance of 1 mm. between the oil-saline interface (cathode) and the anode, the rheobase was on the order of 20 mv. Since the resistance between cathode and anode was directly proportional to the interelectrode distance, increasing this distance to 5 mm., as was done in this work, should result in a fivefold increase in the rheobasic voltage, assuming that the rheobasic amperage is a constant for a fiber type. It follows then, that in terms of the effective stimulating current applied to the tissue, the rheobase values obtained in this work (Table I) are of the same order of magnitude as those obtained by Wright, Coleman, and Adelman (1955).

Since it has been indicated that the threshold of these fibers is on the order of 100 mv. for an interelectrode distance of 5 mm., there appears to be no conflict between the rheobase values in Table I and the "safety factor" concept. Since it takes a rheobasic voltage comparable to the maximum voltage developed by the spike (100 mv.) to stimulate a spot on the nerve 5 mm. away from the current source, it seems very likely that a shorter duration current (the spike is about 1 msec. in duration) of the same voltage would be able to stimulate a region of the nerve membrane some millimeters in advance of the active current source. In terms of current flow, the region in advance of the impulse would be the source and the impulse would be the sink.

That there is some underlying rhythmical process in nerve seems quite probable in view of results presented by Adelman (1956 *a*). In a sense, the local potential may act much like a "pacemaker," in which its frequency, amplitude, and damping regulate the production of the action potential. Recently, Wright and Adelman (1956) showed that when a normally repetitively firing axon becomes non-repetitive the local response is shortened to

appear similar to the normal local response of a normally non-repetitively firing fiber. Wright and Coleman (1954) have revived the concept developed earlier by Arvanitaki (1943) and Brink, Bronk, and Larrabee (1946) that the local impulses are probably the "pacemakers" for the repetitive spike discharge. Small potential oscillations were recorded across the membrane of the squid giant axon by Marmont (quoted by Cole, 1949), and by Hodgkin and Huxley (1952). Usually associated with the action potential and preceding it to some extent was a small potential change referred to by Hodgkin (1938) as the "local potential" and by Wright and Coleman (1954) as the "initial potential." An applied current can be thought of as a condition giving rise to a local potential (Hodgkin and Rushton, 1946) which then triggers the spike or action potential. In the case in which low amplitude potential oscillations resulted from cathodal depression these may have been brought about by the applied current depolarizing the membrane to such an extent that each successive repolarization against the continually applied current was less successful in developing the local potential to its trigger value. This can be considered analogous to an overly increased sodium inactivation (Hodgkin and Huxley, 1952). The possibility that increased current strengths increased the effective cathodal area was always present. However, Adelman (1956 *b*) has shown that as an applied cathode became more diffuse with respect to an applied anode, single muscle fibers became more repetitive to constant current stimulation.

The characterization of the excitabilities of the three types of fibers by the use of real time constants is unwarranted perhaps. On manipulation of the two factor equations to produce a critically damped state, it is found that τ_1 must equal τ_2. However, to produce oscillations the time constants must be conjugately complex. Thus by including real values for τ_1 and τ_2 in the two factor equations the slightly underdamped excitable process is approximated with an overdamped curve. The values of these constants then are only a substitute for the "true constants" which remain complex (*cf.* Cole, Antosiewicz, and Rabinowitz, 1955 p. 500). Certainly, both the excitatory states and the local potentials determined in this work were mildly oscillatory, and therefore the estimated real constants are merely crude approximations.

The author desires to acknowledge his indebtedness to Dr. E. B. Wright for his helpful guidance, encouragement, and stimulation throughout the course of this work.

SUMMARY

1. Three anatomically different types of lobster motor axons have been shown to possess different excitabilities under identical conditions.

2. The type, openers, which fired the longest trains of repetitive responses

also had the lowest rheobase values, the longest utilization times, and the least accommodation.

3. A relationship between the time course of the local potential and the time course of the excitatory state has been disclosed.

4. The characterization of the excitatory state by simple two factor equations has been discussed and found to be only approximate since both the excitatory state and the local response tend to be somewhat oscillatory.

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THE EFFECT OF CHANGES OF ENVIRONMENT ON THE ELECTRICAL AND IONIC PATTERN OF MUSCLE

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(Received for publication, May 14, 1956)

In recent years the interrelationship between the ionic pattern and the electrical properties of nerve and muscle has been the subject of much inquiry (7, 14); for other references, see Grundfest *et al.* (10). The separation of Na^+ and K^+ by the living cell is held to result from a Donnan type distribution maintained by a metabolically mediated extrusion of Na^+ (1, 3, 30). The resting potential is said to arise from this unequal distribution of ions, particularly the K^+ gradient, in a fashion predictable by the Nernst equation. The effect of Na^+ and Cl^- on this potential has been precisely predicted by Goldman (9).

The active phase of nerve and muscle is ascribed to a specific alteration in the permeability of the membrane first to Na^+ ions and then to K^+ ions. The membrane is discharged to a value which is close to the Na^+ diffusion potential.

The validity of the Donnan theory of K^+ retention has been questioned by many authors. (For bibliography, see Shaw and Simon (25).) The nature of the electrical phenomenon has also been held in doubt (10, 11, 23). However, the mathematical treatment of these latter authors, in particular, has been based on the assumption of a Donnan distribution of K^+, with a Na^+ "pump." It would consequently seem of prime importance to test these basic assumptions, so that future work will not be based on doubtful premises.

Apparatus and Methods

The resting and action potentials were measured with 3 molar KCl microcapillary liquid junctions. Tip diameter was about 0.5 μ and electrical resistance varied from 10 to 20 megohm.

The recording equipment consisted of an input cathode follower electrometer (6 AK 5) with a second cathode follower as a buffer stage. The Ag/Ag Cl electrode fitted into a clip attached to the end of the grid pin of the input valve. The input tube was placed in a double shielded can in the same manner as described by Hodgkin and Katz (16). The output from the input stage was connected to two D.C. amplifiers in parallel. One was used to drive a chart recorder for accurate reading of all D.C. levels and the second was connected to a single beam cathode ray oscilloscope.

Voltage calibration was carried out with precision resistors and a standard cell. A fixed voltage (90 mv.) could always be introduced through the indifferent electrode. During an experiment calibration was carried out at regular intervals. A multivibrator circuit was used for time calibration.

A square wave voltage generator was used for stimulation. Its output was isolated from the bath by a special transformer. This generator also provided a square pulse which could be introduced through the indifferent electrode to check the input time constant of the apparatus with the electrode in position, and hence the resistance of the tip. The time constant varied from 75 to 150 μ sec. Low resistance Ag/Ag Cl electrodes were used at both liquid-metal interfaces. Movable platinum electrodes were used for stimulation.

All the manipulation was carried out under microscope control. The muscle was placed in the bottom of a perspex dish on the stage of a Zetopan microscope. The input head with its attached microtip was held fixed at an angle of 45°. Movement of the microscope stage in 3 dimensions was available and was found satisfactory for impalement of the fiber.

The sartorius muscle of the toad *Bufo marinus* was used throughout this work. Muscles were dissected with great care, and all insertions were left intact. The fibers lying on the top 4 to 5 layers were studied. Unfortunately, this muscle in the toad is thoroughly invested with connective tissue, which varies considerably among different specimens and at different times of the year. At times it is "stringy" and difficult to penetrate with all but the sharpest tips. At other times it is "sticky" and seems to block tips frequently. This problem did not arise in studies on several varieties of frogs, where connective tissue appeared to be looser and superficial. However, frogs were not available in the numbers necessary for the ionic analyses.

The ionic analyses were carried out using a Beckman flame spectrophotometer, and the methods used were similar to those described previously (25). In all experiments concerned with ionic changes 12 pairs of muscles were used. Mean values were taken, and the significance of differences between control and treated determined by a "*t*" test. Controls were in all cases companion muscles and, unless otherwise stated, were immersed in normal Ringer for a period of time equivalent to that of the treated. In all tables of ionic content the wet weight figures have been referred to the intracellular level. Consequently a correction has been made for the effect of alteration of the composition of the extracellular fluid. This effect has not been allowed for in dry weight figures, which will include alterations in extracellular composition as well as intracellular changes. The extracellular space has been taken as 15 per cent, except in muscles showing swelling of more than 15 per cent of the total volume. In these cases the extracellular volume has been assumed to be 10 per cent. No correction has been made for the distribution of solids between the two phases.

The justification for assuming 15 per cent extracellular space has been discussed previously (25), when it was pointed out that the maximum value of the extracellular space in low Na^+ muscles was set at that value which would give zero intracellular Na^+ (a negative value being impossible). This maximum value was of the order of 10 per cent. The maximum value which was found in low Na^+ muscles has been confirmed in muscles of low Cl^- content. Plasma Cl^- and muscle Cl^- have been estimated in the same animal, and the lowest muscle Cl^- obtained was 10.6 m.eq./

kg., with a plasma Cl of 86.8 m.eq./liter. The maximum extracellular space in this example, assuming zero intracellular Cl, would be 12.2 per cent (unpublished results). This value agrees well with that calculated by Fenn *et al.* (5), using the same method; viz., 14.5 per cent.

The fact that a similar upper limit to the extracellular space in some muscles is set by two different methods is some justification for choosing a value around 15 per cent. Undoubtedly the extracellular space is a variable, but, if in some muscles it has a maximum value of 10 per cent (it is natural to suppose that some muscles would have a value lower than this), then it is reasonable to assume a mean of 15 per cent. A value as high as 30 per cent could possibly be achieved by a few muscles.

Figures for bioelectric potentials were obtained from the mean of many penetrations, and are shown ± the standard error, with the number of observations given in parentheses. Muscles were placed in the test solutions for at least 1 hour before the listed potentials were obtained. Although the potentials reached a constant value after the first 10 minutes, ionic equilibrium throughout the muscle would not be complete in less than 1 hour. Surface fibers, in which potentials were recorded, would achieve a steady state more rapidly. Muscles were analyzed for Na^+ and K^+ at the end of each experiment.

Ringer solutions used have been described by Shaw, Holman, and Mackenzie (24), and were buffered with phosphate or bicarbonate. To maintain the bicarbonate Ringer at physiological pH it is necessary to aerate continuously with carbogen (5 per cent CO_2 in oxygen). It was not always convenient to do this when working with microtips, so most of this work was carried out with phosphate-buffered Ringer. In low Na Ringer the osmotic pressure was made up with sucrose.

RESULTS

The Effect of Bicarbonate and Phosphate Buffers on the Resting Potential and the Ionic Pattern.—The resting potential (R.P.) measured with the sartorius muscle immersed in phosphate Ringer gave a value of 92 mv. ± 0.5. A series of impalements in bicarbonate Ringer gave 94 mv. ± 0.5. When CO_2 was bubbled through phosphate Ringer (the pH did not fall below 6.8) a small but statistically insignificant increase in the level of the resting potential was found. These results are at variance with those of Desmedt (4) who found a lower resting potential in bicarbonate Ringer. Ling and Gerard (20) had also described a depolarizing effect of CO_2 on frog muscle.

The effect of these buffers on the Na^+ and K^+ content of the muscle was determined by the usual procedure. Six pairs of muscles were used, and one of each pair was soaked in either of the two solutions for 4 hours. On analysis no significant difference in Na^+ and K^+ content was found between the two groups.

The Effect of Hydrogen Ion Concentration.—A series of estimations of the R.P. were carried out in phosphate Ringer, the pH of which was varied between 6.5 and 8.5. Solutions were checked on a Jones pH meter before addition and after removal from the tissue bath. No significant change in R.P.

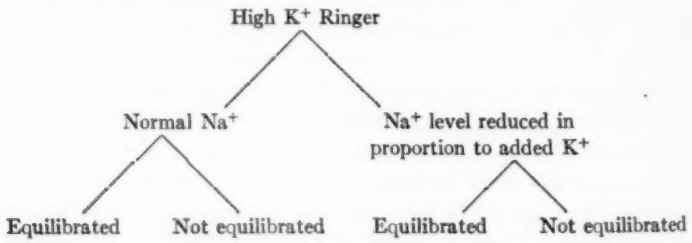
was observed within this range of hydrogen ion concentration, either by the examination of scatter diagrams of individual experiments or from calculation of mean values. When, however, the hydrogen ion concentration was increased to pH 5.8 the R.P. fell off rapidly, and after 10 minutes an average value of 60 mv. was obtained.

The effect of hydrogen ion concentration on the ionic content of muscle has been reported elsewhere (25) and is in distinction to the findings with the intracellular electrode. The graph of K^+ content against pH shows an increase in K^+ level with decreasing hydrogen ion concentration, and shows no marked alteration in shape in the region of pH 5.8. The Na^+ curve passes through a minimum at pH 7.2, and rises continuously at higher and lower hydrogen ion concentrations.

The Effect of Alteration of the External K Level.—The accumulation of K^+ in the cell has in the past been held to be due to a Donnan distribution (1, 3), which resulted in a potential predicted by the Nernst or Goldman equations (9). This concept rests on two experimental procedures. First, Conway found that alteration in the level of external K^+ resulted in an alteration in internal K^+ such as could be theoretically predicted. Second, it has been found (2, 13, 17, 20, 22) that an alteration in the external K^+ resulted in a concomitant variation in resting potential. In these experiments, and in others of a similar nature, no analyses were made to determine the ratio K^+_{in}/K^+_{out}, indeed it was assumed that K^+_{in} did not change, although Conway has shown that this was not so.

The problem has been reinvestigated, as our earlier results (29) had shown that if a muscle were soaked in K^+-free solution there was no change in the internal K^+ compared with the companion muscle in normal Ringer. This finding is, of course, at variance with Conway's theory.

The plan of our experiments is set out in the diagram below:



The experiments have been divided into two series. In one series the Na^+ content of the Ringer has been reduced by an amount osmotically equivalent to the added K^+ (the Cl^- remaining constant), and in the second series the Na^+ content has been held constant and the K^+ and Cl^- content raised. The effects of this alteration in tonicity are reported below. The procedure

was further subdivided so that (*a*) the muscles were removed from the animal and placed immediately in the high K^+ Ringer (non-equilibrated) and (*b*) they were placed in normal Ringer for 2 hours, and then removed to the high K^+ Ringer (equilibrated) for a further 3 to 4 hours. Control muscles were analyzed at the end of the equilibration period (2 hours). Muscles placed in normal Ringer lose K^+ during the first 2 hours, and then tend to achieve a steady state with regard to their ionic content. It was our intention to see whether we could influence this loss, or alter the steady state level of the cell. It had been shown (1, 19, 21) that there was a "threshold" level of K^+ in the Ringer which would prevent this initial leakage.

The results of our experiment are set out in Table I. In the first part of this table the muscles were placed in Ringer which had been rendered hypertonic by the addition of KCl to normal Ringer. The effects of these solutions are shown with and without previous equilibration. It will be seen that there has been no volume change, although the tonicity of the solution has been increased by as much as one-third. Raising the external K^+ level to 12.5 and 25 m.eq./liter has not prevented the leakage of K^+ from the cell, nor caused a return of K^+ to the cell, in the non-equilibrated and equilibrated series respectively. At 50 m.eq./liter (18 times normal) there has been a moderate increase in the K^+ level compared with muscles in normal Ringer, in both series.

There has been no change in the Na^+ level of the tissue, save for a slight drop in the unequilibrated series at the highest level of external K^+.

The second part of Table I shows the results of experiments in which, as KCl was added to the Ringer, an equivalent amount of NaCl was subtracted. These findings differ in three respects from those of the previous series.

First, there was a definite increase in volume in the non-equilibrated series of about 17 per cent, when the Na^+ in the Ringer was lowered to 100 m.eq./liter or less. The degree of swelling was less in the equilibrated series, being 11.7 per cent as compared with 17.0 per cent.

Second, when the results are considered on a wet weight basis there has been no change in the internal K^+ level, save for a slight gain at the highest external K^+ level. As the muscles had increased in volume there was, however, an actual migration of K^+ across the membrane, which was evident from the results referred to a dry weight basis.

The third point brought out in these experiments is the effect of high K–low Na Ringer on the internal Na^+ level. In both series there was a marked decrease in the Na^+ content of the cell, which was greater than would have been expected from the actual decrease in the Na^+ level of the Ringer. This effect is shown in Table II. The normal ratio Na^+_{out}/Na^+_{in} is approximately 3, but under these experimental conditions it has increased to as much as 7.6.

A direct comparison of the effects of normal and reduced Na^+ in high K^+ Ringer is shown in Table III. The results, which are derived from 6 pairs of muscles, confirm those which had been obtained separately above. The

TABLE I

The Effect of High K Ringer on Ionic Content of Muscles

			Na level of Ringer not altered				
K in Ringer, *m.eq./liter*			Equilibrated		Not equilibrated		
			12.5	50	12.5	25	50
Volume change, *per cent*			+1.7	+0.6	+2.3	−1.6	+0.6
Na, *m.eq./kg.*	Wet weight	Control	36.1	43.2	43.0	34.7	29.3
		Treated	35.8	44.0	39.4	30.5	24.2*
	Dry weight	Control	260	302	272	246	230
		Treated	259	323	267	216	200
K, *m.eq./kg.*	Wet weight	Control	86.3	67.7	90.0	80.3	69.0
		Treated	85.2	81.4*	91.2	84.9	94.4*
	Dry weight	Control	379	313	385	353	288
		Treated	386	413*	392	380	438*

			Na level of Ringer reduced proportionately				
K in Ringer, *m.eq./liter*			Equilibrated		Not equilibrated		
			25	50	12.5	25	50
Volume change, *per cent*			+0.7	+11.7	+4.4	+15.2	+17.0
Na, *m.eq./kg.*	Wet weight	Control	38.5	37.6	44.8	51.4	40.1
		Treated	30.8*	16.7*	34.8*	39.1*	15.8*
	Dry weight	Control	284	274	356	371	380
		Treated	209*	105*	306*	219*	175*
K, *m.eq./kg.*	Wet weight	Control	79.9	90.2	80.0	81.9	85.1
		Treated	85.8	99.0*	91.2	86.7	102*
	Dry weight	Control	372	400	449	350	365
		Treated	423*	528*	497*	478*	601*

In this and other tables of ionic content wet weight values are calculated at intracellular level on a basis of 15 per cent extracellular volume. Each figure is the mean of at least 12 observations. Only figures marked with an asterisk are significantly different from control ($P < 0.05$). K content of Ringer and volume changes refer to treated muscles only.

muscles in the low Na Ringer increased in volume by 15.2 per cent. There was no difference in the K^+ content on a wet weight basis, but dry weight figures indicate that some K^+ had crossed the membrane in the low Na^+

muscles. (Dry weight figures in this series may be compared directly, as the extracellular K^+ included in the figures is the same in both cases.) The latter muscles also showed an extrusion of Na^+, increasing the ratio Na^+_{out}/Na^+_{in}, as was found previously.

Bioelectric Changes.—The concept of a Donnan type distribution of K^+ has led to the belief that the resting potential is a "K^+ diffusion potential," modified by the effect of Na^+ and Cl^- permeability (9). The effect of

TABLE II

The Effect of High K—Low Na Ringer on Ratio $\frac{Na_{out}}{Na_{in}}$

K in Ringer	Na in Ringer	Ratio $\frac{Na_{out}}{Na_{in}}$	
		Equilibrated	Not equilibrated
m.eq./liter	*m.eq./liter*		
12.5	117.5	—	3.6
25	105	2.5	4.6
50	80	3.3	7.6

TABLE III

A Comparison of the Effect of High K–Low Na Ringer with High K–Normal Na Ringer on Ionic Content

Na in Ringer	Volume change	Na, *m.eq./kg.*		K, *m.eq./kg.*	
		Wet weight	Dry weight	Wet weight	Dry weight
m.eq./liter	*per cent*				
130	Nil	44.4	331	96.8	459
80	+15.2	20.0	192	93.4	559*

One muscle was placed in Ringer containing K 50 m.eq./liter and Na 130 m.eq./liter, and the companion placed in K 50 m.eq./liter and Na 80 m.eq./liter, for 4 hours. The difference in wet weight K found is not significant.

* Significant.

alteration of the external K^+ level on the R.P. has been one of the strongest arguments in the justification of this belief.

This experiment has been repeated by us, and the results of the measurements of the R.P. in concentrations of K^+ varying from 0 to 54 m.eq./liter are given in Fig. 1. At a concentration of more than 13.5 m.eq./liter K^+ A.P. were obtained only during the first few minutes. After this time the muscle was inexcitable but the R.P. remained constant for several hours. It will be noted that it is only after this point that there is a linear relationship between the R.P. and the log of the external K^+ concentration.

This investigation was now extended along the lines set out in the plan

on page 266. The results may be seen in Table IV. The following points should be noted: (1) The observed R.P. are always more than 35 per cent higher than the potential predicted from the Nernst equation on the basis of the ionic analyses which were carried out at the end of each experiment. (2) The observed R.P. were the same irrespective of whether the muscles had been previously equilibrated in normal Ringer or not. (3) In high

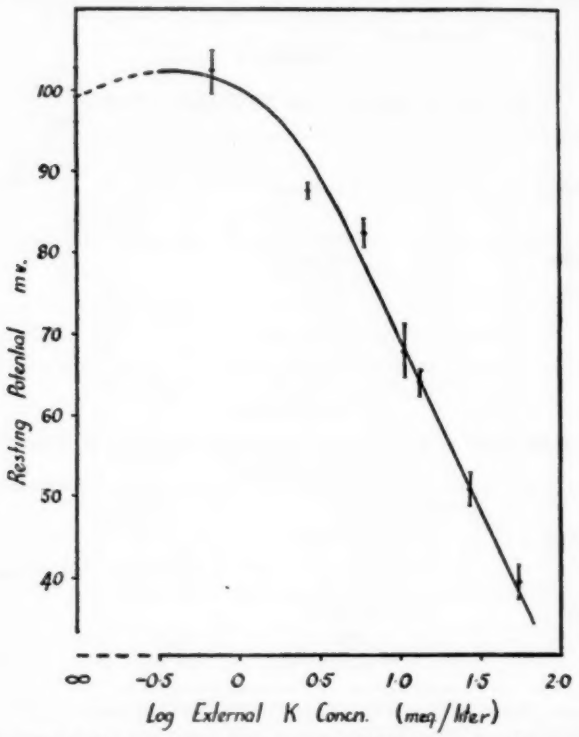


FIG. 1. The effect of variation in the external K^+ level on the resting potential. The vertical lines represent confidence limits at 5 per cent. There was a minimum of 30 readings for each point.

K^+ Ringer there was no significant difference in R.P., whether Na^+ was normal or reduced by an amount equivalent to the added K^+.

The ratio Na^+_{out}/Na^+_{in} was not always greater in reduced Na^+ solutions, as would have been expected from the results presented in the previous section. This may have been due to the fact that these results were collected from experiments carried out over several months, when the initial ionic content of the muscles would be expected to vary (see reference 26). In order to obviate this difficulty, experiments were performed using paired muscles. One member was placed in normal Na–high K Ringer, and the companion

muscle was placed in low Na–high K Ringer. The results of these experiments are set out in Table V. It will be seen that in 4 of the 5 experiments the low Na–high K Ringer-treated muscles showed an increase in Na^+ gradient. The

TABLE IV

The Effect of High K Ringer on the Resting Potential

K in Ringer	Na in Ringer	Resting potential		K	Na	Ratio Na_{out}/Na_{in}
		Calculated	Observed			
m.eq./liter	*m.eq./liter*			*m.eq./kg.*	*m.eq./kg.*	
Not equilibrated						
13.5	130	45.8	57.4 ± 1.2 (15)	83.8	45.0	2.9
		37.1	52.4 ± 1.7 (5)	60.0	54.9	2.4
13.5	117	44.3	53.2 ± 2.2 (7)	80.2	49.0	2.4
		21.6	57.0 ± 1.8 (9)	51.2	55.0	2.1
30	130	27.2	36.9 ± 0.7 (29)	87.5	51.8	2.5
30	100	27.0	36.3 ± 0.26 (30)	84.6	22.3	4.6
50	130	18.9	31.5 ± 0.6 (15)	106	23.0	5.7
50	80	21.0	33.0 ± 1.8 (9)	115	11.5	6.9
Equilibrated						
27	130	36.3	38.5 ± 0.4 (20)	114	57.1	2.3
30	130	28.6	38.0 ± 1.1 (29)	93.4	47.1	2.8
32	130	29.6	37.6 ± 0.7 (11)	105	40.0	3.2
		29.5	40.0 ± 0.9 (17)	104	56.0	2.5
32	98	26.3	35.0 ± 0.6 (12)	92.0	23.1	3.3
		27.4	32.1 ± 0.6 (21)	96.5	15.3	6.5
30	100	27.6	37.7 ± 0.4 (31)	89.6	34.6	2.9
50	130	19.4	32.5 ± 0.4 (26)	108	23.6	5.5
		18.4	29.3 ± 0.5 (23)	104	20.0	6.5
50	80	13.3	28.7 ± 0.5 (21)	84.6	15.7	5.1
		9.5	31.0 ± 1.1 (21)	72.8	14.9	5.4

In this and subsequent tables bioelectric potentials are shown ±S.E. The number of observations is given in parentheses. Ionic contents are referred to intracellular levels.

drop in R.P. in this series compared with that found in the normal Na–high K-treated muscles is small, but was statistically significant at the 5 per cent level.

The significance of these results for the prediction of the R.P. by the Goldman equation will be referred to in the discussion.

The Effect of Alteration of the External Na Level.—It would appear that up to the present time there has not been an adequate investigation of the alterations in internal Na^+ in response to alterations of the level of this ion in the external environment. Fenn (5) did not calculate his Na^+ contents on an intracellular basis. If one makes this correction then his results are in agreement with those listed below. This would seem of great importance in the elucidation of the nature of Na^+ extrusion from the cell, and also in determining the relationship between the Na^+ gradient and the A.P. It is held that

TABLE V

The Effect of Alteration in Na Gradient on the Resting Potential in High K Ringer

K 46 m.eq./liter (× 17).

Na^+ in Ringer	Resting potential	Na, *m.eq./kg. wet weight*	K, *m.eq./kg. wet weight*	Ratio
130	29.1 ± 0.6 (30)	38.2	82.2	3.4
85	29.0 ± 0.8 (25)	10.3	102	8.2
130	32.8 ± 0.8 (26)	45.9	63.0	2.8
85	29.5 ± 0.6 (28)	9.9	96.2	8.6
130	33.6 ± 0.6 (29)	50.4	99.5	2.6
85	32.5 ± 0.8 (25)	17.8	96.5	4.8
130	31.9 ± 0.7 (28)	57.8	103	2.2
85	28.5 ± 0.9 (24)	41.4	92.2	2.1
130	31.5 ± 0.7 (31)	41.0	96.7	3.2
85	30.0 ± 0.5 (31)	10.6	101	7.6

Ionic contents refer to intracellular levels. Ratio means $\frac{Na_{out}}{Na_{in}}$.

during the passage of the spike the membrane is discharged to a value close to that of the "Na^+ diffusion potential." This concept has rested largely on experiments relating the alteration in external Na^+ to the rise of the overshoot (15, 16) (see also reference 4). It has been assumed in these experiments that the internal Na^+ level remains constant.

We have investigated fully the effect of alteration of external Na^+ on the ionic and potential patterns in the same manner as was done with K^+, *i.e.* muscles were soaked in high and low Na^+ Ringer, with and without previous equilibration in normal Ringer.

Ionic Changes.—The external Na^+ level has been varied between 45 and 650 m.eq./liter. In low Na^+ solutions the osmotic pressure has been made up

with sucrose. High Na^+ solutions (> 130 m.eq./liter) were of necessity hypertonic.

Low Na Ringer (Unequilibrated).—As will be seen from Table VI muscles placed in reduced Na Ringer lose Na^+ in proportion to the decrease in the Na^+ level of the external solution. Thus, after 2 to 4 hours' soaking, when the muscle has reached a steady state, the ratio Na^+_{out}/Na^+_{in} is the same or slightly greater than that of the other member of the pair which was soaked in normal Ringer. There has been no concomitant change in K^+ level of the muscle.

Low Na Ringer (Equilibrated).—When a muscle is soaked in Ringer it tends to gain Na^+, until a steady state level is achieved, with a ratio Na^+_{out}/Na^+_{in} approximately equal to 3. If this muscle is now removed to a Na^+-deficient

TABLE VI

The Effect of Reduced Na Ringer on Ionic Content

	Equilibrated				Not equilibrated			
Na in Ringer, *m.eq./liter*	43.4 (× 0.33)	Ratio	65 (× 0.50)	Ratio	43.4 (× 0.33)	Ratio	65 (× 0.50)	Ratio
Na, *m.eq./kg.*								
Control	52.0	2.5	39.2	3.3	46.1	2.8	31.9	4.5
Treated	21.2	2.0	21.5	3.0	12.6	3.4	12.9	5.0
K, *m.eq./kg.*								
Control	87.0		82.0		81.2		93.2	
Treated	86.0		90.0		85.0		92.6	

Ratio means ratio $\frac{Na_{out}}{Na_{in}}$.

solution Na^+ is extruded from the cell, against a concentration gradient, until the Na^+ ratio is almost completely reestablished. These results are set out in Table VI. The control group of 6 muscles was always removed from the Ringer solution for analysis at the end of the equilibration period. As in the unequilibrated series there was no change in K^+ levels.

The relation of this Na^+ extrusion to the Steinbach (30) "pump" is discussed in two other papers (27, 28).

High Na Ringer (Equilibrated and Unequilibrated).—The results of soaking muscles in high Na Ringer are set out in Table VII. It will be seen that as the osmotic pressure of the external solution is increased there is a progressive shrinkage, which does not however, exceed 22 per cent, even when the Na^+ concentration has been increased by 500 per cent. It is also evident that the internal Na^+ level has been elevated in proportion to the rise in external Na^+. As was found in the low Na^+ series the ratio Na^+_{out}/Na^+_{in} has remained

constant at a value of approximately 3. In Ringer with more than 390 m.eq./liter Na^+ the muscles became bunched up and unexcitable after about half an hour.

TABLE VII

The Effect of High External Na on Ionic Content of Muscles

	Unequilibrated									
Na in Ringer, *m.eq./liter*.....	156 (× 1.2)	Ratio	173 (× 1.33)	Ratio	390 (× 3)	Ratio	520 (× 4)	Ratio	650 (× 5)	Ratio
Volume change, *per cent*.....	−5.5		−6.8		−15.0		−17.7		−22.9	
Na, *m.eq./kg. wet weight*										
Control.....	49.5	2.8	47.0	2.9	44.0	3.1	36.0	3.9	36.5	3.8
Treated.....	62.5	2.7	67.0	2.7	121	3.4	138	4.0	222	3.1
K, *m.eq./kg. wet weight*										
Control.....	81.0		77.5		76.5		91.0		70.8	
Treated.....	76.5		71.3*		51.6*		58.0*		4.5*	

	Equilibrated						
Na in Ringer, *m.eq./liter*.....	260 (× 2)	Ratio	650 (× 5) (3 hrs.)	Ratio	650 (× 5) (5 hrs.)	Ratio	
Volume change, *per cent*.....	−14.4		−22.4		−15.9		
Na, *m.eq./kg. wet weight*							
Control.....	52.8	2.8	25.8	5.0	21.0	6.2	
Treated.....	83.0	3.3	177	3.7	234	2.8	
K, *m.eq./kg. wet weight*							
Control.....	79.7		95.0		101		
Treated.....	57.5*		37.6*		16.0*		

K contents marked with an asterisk are significantly different from control ($P < 0.05$).

Ratio means $\frac{Na_{out}}{Na_{in}}$.

As the Na^+ level of the Ringer is raised there is a concomitant decrease in the K^+ level of the cells. This is just evident when the Na^+ is increased by 33 per cent, and results in a loss of 90 per cent of the cellular K^+ when the external Na^+ is increased by 500 per cent. In some muscles at this level of

Na^+, there was no K^+ intracellularly. Thus there had been an extrusion of K^+ against a concentration gradient. A similar K^+ extrusion was previously noted by us (24).

Bioelectric Changes.—The effect of alteration of external Na^+ level on the height of the overshoot has been investigated by several authors over a range of concentrations from less than normal Ringer to 120 per cent the normal level (14–16, 22). They have reported a decrease in the height of the overshoot in

TABLE VIII

The Effect of Reduced Na Ringer on the Resting Potential and Overshoot

Unequilibrated series.

Experiment	Time in low Na Ringer	Potential				Analysis			
		Control		Treated		Control		Treated	
		R. P.	Overshoot	R. P.	Overshoot	Na, m.eq./kg.	K, m.eq./kg.	Na, m.eq./kg.	K, m.eq./kg.
	min.								
1	0–10	82.3±0.7 (17)	36.3±0.8 (17)	78.8±1.3 11)	23.5±2.0 (11)				
				80.5±1.4 (12)	27.3±1.1 (12)	47.0	93.6	29.2	84.6
2	0–10	92.6±1.6 (12)	33.3±1.8 (12)	86.2±1.2 (8)	26.0±2.8 (8)				
				86.4±1.2 (18)	19.5±1.3 (18)	42.6	100	24.6	108
3	0–10	94.6±0.7 (20)	33.6±1.1 (20)	85.7±0.9 (14)	22.4±2.1 (14)				
	60–90			86.7±0.7 (29)	20.1±1.3 (29)	54.0	79.5	29.3	103
		Na 46.0 m.eq./liter (× 0.33)							
1	0–10	—	—	85.1±1.3 (15)	22.5±0.9 (15)				
	60–90			85.7±0.8 (15)	17.1±2.2 (15)	24.5	80.0	9.2	95.0
2	0–10	91.3±1.6 (16)	42.7±1.7 (16)	77.0±1.6 (16)	15.4±1.6 (16)				
	60–90			83.8±1 0 (22)	14.6±1.4 (22)	59.5	91.5	51.6	83.4
3	0–10	92.5±0.8 (16)	36.8±1.3 (16)	81.3±1.1 (23)	21.8±1.3 (23)				
	60–90			79.6±0.9 (20)	15.2±6.9 (20)	51.0	85.0	26.7	97.2

Ionic contents refer to intracellular levels.

reduced Na^+ Ringer, with a slope of 58 mv. for a tenfold decrease in Na^+ level. A slight increase in the overshoot in 120 per cent Na Ringer was also noted.

We have investigated the effect of alteration of the external Na^+ on the R.P. and A.P. with a procedure similar to that used in the previous section.

Low Na Ringer (Unequilibrated).—The sartorius muscle was removed from the animal and immediately placed in reduced Na Ringer. R.P. and A.P. were recorded at once, and at intervals over the next 90 minutes. Control readings on the paired muscle were taken after 2 hours' soaking in normal Ringer. At the end of the experiment both muscles were analyzed. The results are set out in Table VIII.

It will be seen that the R.P. is several millivolts lower in reduced Na Ringer, although there has been no alteration in the intracellular K^+ level. The overshoot fell as soon as the muscle was placed in low Na Ringer, and remained

TABLE IX

The Effect of Reduced Na Ringer on the R.P. and Overshoot

Equilibrated—Na 65 m.eq./liter (× 0.5).

Experiment	Time in Ringer	Potential			Analysis			
		Resting potential	Overshoot		Control		Treated	
			Found	Calculated	Na, m.eq./kg.	K, m.eq./kg.	Na, m.eq./kg.	K, m.eq./kg.
1	2 hrs. normal Ringer (*a*)	89.5 ± 1.4 (8)	36.0 ± 2.6 (8)	30.7				
	0–10 min. half Na Ringer (*b*)	85.4 ± 1.1 (10)	29.6 ± 1.5 (10)	13.8				
	60–90 min. half Na Ringer (*c*)	87.9 ± 1.7 (10)	28.1 ± 1.6 (10)	20.2	38.4	89.4	29.3	95.0
2	(*a*)	87.9 ± 1.3 (13)	40.6 ± 1.9 (13)	30.0				
	(*b*)	84.1 ± 0.9 (10)	35.7 ± 1.5 (10)	12.6				
	(*c*)	84.5 ± 1.7 (7)	31.1 ± 1.1 (7)	22.8	39.7	106	26.3	94.0
3	(*a*)	89.7 ± 1.9 (3)	42.2 ± 3.2 (3)	33.9				
	(*b*)	88.6 ± 1.1 (12)	30.2 ± 2.2 (12)	16.4				
	(*c*)	90.0 ± 1.9 (10)	28.0 ± 2.4 (10)	24.1	33.9	104	25.0	100
4	(*a*)	90.7 ± 1.8 (9)	40.6 ± 2.5 (8)	44.6				
	(*b*)	84.1 ± 1.5 (12)	23.3 ± 1.9 (11)	27.2				
	(*c*)	81.8 ± 1.5 (10)	21.1 ± 2.5 (10)	36.2	22.1	90.0	15.4	96.0
5	(*a*)	90.8 ± 3.1 (9)	37.9 ± 2.1 (9)	34.7				
	(*b*)	81.6 ± 1.8 (11)	29.4 ± 1.3 (11)	17.3				
	(*c*)	87.0 ± 1.3 (10)	18.5 ± 1.1 (10)	39.2	32.8	64.2	13.7	85.0

Ionic contents refer to intracellular levels. Calculated overshoot from 58 log $\frac{Na_{out}}{Na_{in}}$.

In (*b*), 0 to 10 minutes in half Na Ringer, Na_{in} is assumed to be equal to that of control muscle.

at this level, or in some cases declined further during the next 90 minutes. In all but one experiment the Na^+ ratio, as determined by analysis at the end of the experiment, remained at the normal value. The results for the height of the overshoot recorded in one-third Na Ringer showed considerable scatter and in some cases the fiber would not "fire" on stimulation.

Low Na (Equilibrated).—The muscle was removed from the animal and

placed in normal Ringer for 2 hours. During this period measurements were made on R.P. and A.P. The Ringer was then replaced with half Na Ringer and potentials were again measured for a further 90 minutes. The paired

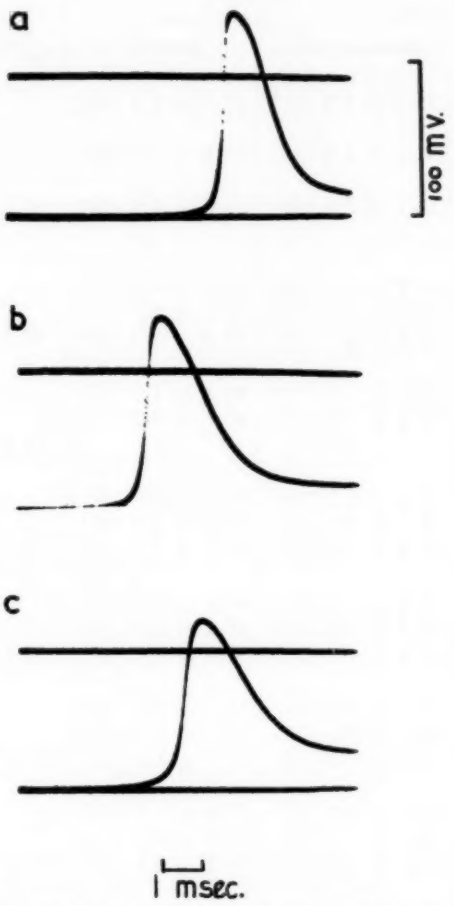


FIG. 2. The effect of half Na^+ Ringer on the configuration of the action potential. (*a*) A.P. in normal Ringer; (*b*) A.P. within 5 minutes of placing in half Na^+ Ringer; (*c*) A.P. after 90 minutes in half Na^+ Ringer. In this and subsequent photographs the upper horizontal line represents zero potential.

muscle was soaked in Ringer for 2 hours, and then removed for analysis. The extent of the Na^+ extrusion into the half Na Ringer was gauged from the difference in analysis of the two muscles.

The results of 5 typical experiments may be seen in Table IX. When the

TABLE X

The Effect of High Na Ringer on the Resting Potential and Overshoot

Equilibrated series. Na 156 m.eq./liter (× 1.2).

Time in Ringer	Potential		Analysis		
	Resting potential	Overshoot	Na, *m.eq./kg.*	K, *m.eq./kg.*	$\frac{Na_{out}}{Na_{in}}$
2 hrs. in normal Ringer (*a*)	88.2 ± 1.0 (16)	37.3 ± 1.1 (16)			
0–10 min. in high Na (*b*)	86.1 ± 1.4 (18)	33.8 ± 1.4 (18)			
90–120 min. in high Na (*c*)	88.2 ± 0.9 (20)	39.4 ± 0.8 (20)	87.5	93.3	1.8
(*a*)	91.2 ± 1.1 (13)	30.0 ± 1.5 (13)			
(*b*)	87.0 ± 1.6 (12)	30.0 ± 1.7 (12)			
(*c*)	92.7 ± 1.3 (12)	27.0 ± 1.1 (12)	81.0	87.0	2.0
(*a*)	89.4 ± 1.2 (12)	45.8 ± 1.6 (12)			
(*b*)	87.5 ± 0.8 (11)	40.1 ± 0.9 (11)			
(*c*)	—	—	66.0	105	2.4
(*a*)	90.0 ± 0.9 (20)	41.0 ± 0.9 (20)			
(*b*)	89.1 ± 0.8 (14)	41.1 ± 1.3 (14)			
(*c*)	87.8 ± 1.0 (25)	35.4 ± 1.1 (25)	70.5	84.5	2.2
(*a*)	85.9 ± 1.3 (14)	40.4 ± 0.9 (14)			
(*b*)	91.2 ± 0.9 (12)	41.2 ± 1.0 (12)			
(*c*)	89.6 ± 1.0 (10)	36.2 ± 1.6 (10)	72.5	80.4	2.2
(*a*)	84.3 ± 0.8 (18)	41.1 ± 1.0 (18)			
(*b*)	86.0 ± 1.1 (12)	41.3 ± 1.8 (12)			
(*c*)	87.9 ± 0.8 (12)	36.7 ± 1.8 (12)	73.4	80.0	2.1
(*a*)	90.5 ± 0.9 (17)	27.8 ± 1.2 (17)			
(*b*)	89.7 ± 1.1 (13)	30.2 ± 1.4 (13)			
(*c*)	89.0 ± 1.2 (17)	33.5 ± 0.8 (17)	61.2	92.7	2.5
(*a*)	88.3 ± 1.2 (15)	26.5 ± 0.8 (15)			
(*b*)	88.5 ± 1.0 (21)	27.8 ± 0.8 (21)			
(*c*)	87.7 ± 0.9 (19)	32.3 ± 1.2 (19)	90.0	98.5	1.7
(*a*)	91.5 ± 3.3 (8)	36.1 ± 3.4 (8)			
(*b*)	90.4 ± 1.2 (9)	38.5 ± 1.9 (9)			
(*c*)	91.0 ± 1.5 (11)	36.7 ± 1.9 (11)	47.2	104	3.3

Ionic contents are referred to intracellular levels.

TABLE XI

The Effect of High Na on the Resting Potential and the Overshoot Equilibrated

Na in Ringer	Time in Ringer	Potential		Analysis		
		R. P. Resting potential	Overshoot	Na, m.eq./kg.	K, m.eq./kg.	$\frac{Na_{out}}{Na_{in}}$
m.eq./liter						
173	2 hrs. normal	91.7 ± 1.9 (6)	36.6 ± 2.5			
(× 1.33)	0–10 min. high Na	90.7 ± 1.7 (13)	34.6 ± 1.6 (13)			
	120–180 min. high Na	95.3 ± 1.2 (15)	34.8 ± 1.1 (15)	61.0	100	2.85
	2 hrs. normal	91.0 ± 1.4 (9)	38.7 ± 2.7 (9)			
	0–10 min. high Na	89.3 ± 1.8 (14)	37.5 ± 2.4 (14)			
	120–180 min. high Na	86.5 ± 2.6 (11)	34.8 ± 2.3 (11)	68.3	95.4	2.7
195	2 hrs. normal	90.4 ± 1.4 (7)	47.0 ± 1.8 (7)			
(× 1.5)	0–10 min. high Na	90.8 ± 0.9 (17)	46.0 ± 1.0 (17)			
	100–120 min. high Na	91.7 ± 1.0 (11)	36.0 ± 1.5 (11)	66.6	102	2.9
	2 hrs. normal	93.5 ± 1.1 (8)	42.6 ± 0.9 (8)			
	0–10 min. high Na	96.1 ± 1.2 (12)	40.5 ± 2.5 (12)			
	100–120 min. high Na	92.4 ± 0.9 (10)	33.0 ± 1.2 (12)	73.5	94.1	2.8
260	2 hrs. normal	93.6 ± 2.3 (7)	40.3 ± 2.0 (7)			
(× 2.0)	0–10 min. high Na	95.6 ± 1.5 (10)	38.7 ± 2.7 (10)			
	100–120 min. high Na	88.8 ± 1.4 (14)	31.3 ± 1.6 (14)	103	114	2.7
390	2 hrs. normal	94.8 ± 0.9 (9)	37.9 ± 2.1 (9)			
(× 3.0)	0–10 min. high Na	97.2 ± 2.0 (10)	38.3 ± 2.6 (10)			
	120–180 min. high Na	91.4 ± 1.0 (15)	25.3 ± 1.3 (15)	133	73.9	3.1
520	—	—	—			
(× 4.0)	0–10 min. high Na	82.6 ± 1.9 (13)	43.8 ± 1.7 (13)			
	200–240 min. high Na	24.5 ± 1.8 (16)	—	279	18.9	2.0
650	2 hrs. normal	91.3 ± 1.1 (19)	32.3 ± 1.3 (10)			
(× 5.0)	0–10 min. high Na	92.0 ± 2.2 (10)	33.3 ± 1.9 (10)			
	30–40 min. high Na	45.3 ± 4.5 (13)	26.0 ± 3.4 (8)	282	33.5	2.4
	2 hrs. normal	94.7 ± 1.7 (14)	29.1 ± 1.5 (14)			
	0–10 min. high Na	89.2 ± 2.5 (16)	27.3 ± 2.7 (16)			
	30–40 min. high Na	45.4 ± 4.5 (11)	29.9 ± 4.2 (8)	314	39.2	2.2

Ionic content refers to intracellular levels.

Ringer was changed from normal to half Na^+, the Na^+ ratio would initially be half that in normal Ringer. The results show that the overshoot has only decreased by about 25 per cent. During soaking the Na^+ ratio has returned close to its original value; but the overshoot has not altered. Indeed the po-

tential has in all cases declined further by a few millivolts. As was seen in the unequilibrated series there has been no change in the intracellular K^+ throughout these experiments. On the other hand there has been a small but statis-

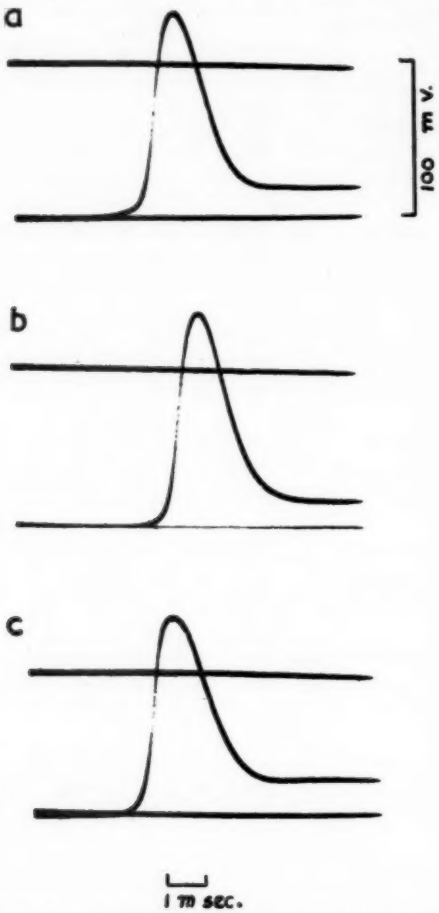


FIG. 3. The effect of $Na^+ \times 2$ Ringer (260 m.eq./liter) on the configuration of the A.P. (*a*) A.P. in normal Ringer; (*b*) A.P. within 5 minutes of placing in high Na^+ Ringer; (*c*) A.P. after 200 minutes in high Na Ringer.

tically significant drop of 3 mv. in R.P. on changing the Ringer, which was partially reversed on further soaking. The measurements of the overshoot have been corrected for the effects of extrafibrillary potentials (26), which were of similar magnitude to those obtained with isolated muscles in normal Ringer. The nature of the extrafibrillary potentials will be discussed fully at a later date. They are similar to the distortions of the action potential de-

scribed by Easton (6). Penetration of the membrane by the capillary electrode was more difficult in low Na Ringer.

We have also compared the configuration of the action potential in normal Ringer with that in half Na Ringer. In some cases (about 10 per cent) there is no difference in configuration; in others the rate of rise is the same, but the duration of the A.P. is longer; and in some cases (about 50 per cent) the foot, or "take off," is slower. See Fig. 2.

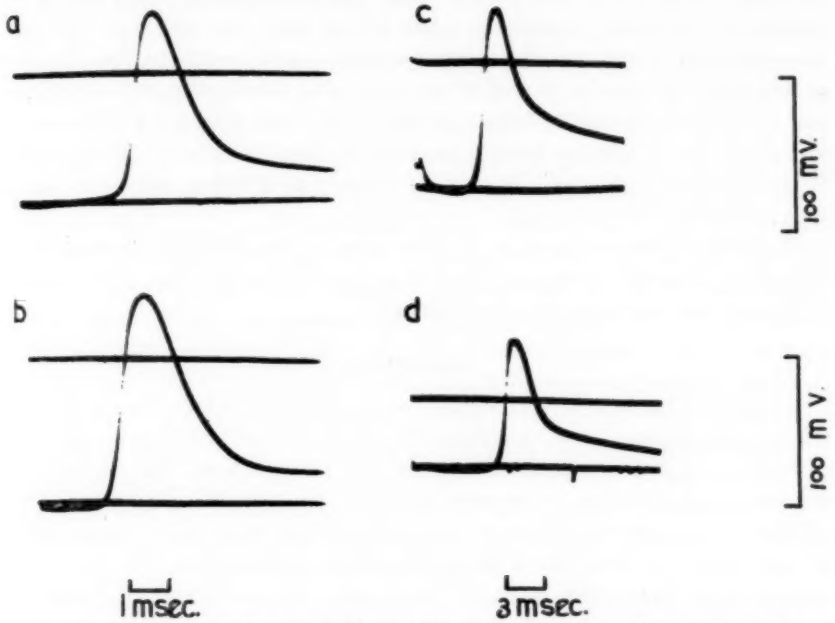


FIG. 4. The effect of $Na^+ \times 5$ Ringer (650 m.eq./liter) on the configuration of the A.P. (*a*) A.P. in normal Ringer; (*b*) A.P. within 5 minutes of placing in high Na^+ Ringer; (*c*) A.P. after 16 minutes in high Na^+ Ringer; (*d*) A.P. after 35 minutes in high Na^+ Ringer.

High Na Ringer (*Equilibrated*).—Other workers (14–16, 22) have found that slight increases in the external Na^+ have resulted in concomitant increases in the A.P. This has been assumed to follow from an increase in the ratio Na^+_{out}/Na^+_{in}, but such an increase has never been demonstrated.

The results obtained on placing the muscle in solutions with excess Na^+ are given in Tables X and XI. At an external level of 156 m.eq./liter Na^+ (120 per cent) the R.P. and overshoot have remained constant at the level recorded in normal Ringer. Potentials were measured over a period of at least 2 hours, and were found to be unchanged. These results were duplicated in 133 per cent Na Ringer, but when the Na^+ concentration was raised to 150

per cent the overshoot tended to fall after 2 hours in the high Na^+ solution. This fall was more marked at higher Na^+ levels, and at levels greater than 300 per cent A.P. were obtained only during the first 30 to 40 minutes in high Na^+. The R.P. remains essentially constant until we reach 300 per cent external Na^+, and beyond this point falls rapidly after the muscle has been in the high Na^+ for more than 25 minutes. It should be noted, however, that even in 500 per cent Na^+ it is possible to obtain R.P. and A.P. of normal height and dimensions during this period. After this initial period when normal potentials are obtained, there is a rapid fall in both R.P. and A.P., and after approximately 5 minutes the muscle bunches, and ceases to fire. The R.P. at this point is between 30 and 40 mv., and it is noteworthy that almost normal A.P. have occasionally been obtained from fibers with a R.P. as low as 35 mv. After the muscle has become unexcitable the R.P. falls to a level of 20 to 30 mv., where it remains constant for as long as 4 hours, although there is then very little K^+ in the cell.

The configuration of the A.P. may be seen in Figs. 3 and 4. Accompanying a decrease in height of the A.P. there is an increase in duration, and a marked increase in the value for the after-potential.

DISCUSSION

In previous papers (25, 29), evidence was presented which tended to refute the hypothesis that the accumulation of K^+ in the cell was due to some type of Donnan mechanism. In particular we were able to show that the complete elimination of K^+ from the external Ringer did not result in the loss of intracellular K^+ greater than that of a control muscle soaked in normal Ringer. In this paper we have investigated the effect of increasing the level of the external K^+. The earlier work of this nature was performed by Boyle and Conway (1). Their results showed that the K^+ entered the cell in proportion to the increase in external K^+, from which they drew the conclusion that the intracellular K^+ level could be attributed to the electrochemical gradient, the product $K^+_{in} \times Cl^-_{in}$, being equal to the product $K^+_{out} \times Cl^-_{out}$.

More recently, Netter *et al.* (19, 21), using a different technique, *i.e.* perfusion of the whole hind limbs rather than immersion of a single muscle, have presented results which substantially confirm those of Boyle and Conway. Our experiments differ from those of the above workers in that we have employed the toad whilst the frog has served as the experimental animal for other workers. In a small series of experiments we have found that the results obtained with the toad could be duplicated with the tissues of the frog *Hyla caerulea*. The effect of alteration of the external K^+ level can be studied in four ways: the additional K^+ may be added directly to the Ringer or an equivalent amount of Na^+ may be removed; the muscle may then be placed in these altered Ringer solutions immediately after dissection (unequilibrated), or after

equilibration in normal Ringer. During the equilibration period there has, of course, been some loss of K^+ and gain of Na^+, until a steady state is reached. Boyle and Conway (1) placed their muscles directly into Ringer from which a constant amount of Na^+ had been extracted, regardless of the alteration of external K^+. Netter *et al.* (19, 21) also perfused their muscles without previous equilibration, but did reduce the Na^+ in proportion to the added K^+. The results of these latter authors will be discussed in a later paper along with our own results obtained with perfusion methods.

Our results are directly opposed to those of Boyle and Conway. We have followed this problem in the four directions outlined above, and we have consistently found that the intracellular K^+ is uninfluenced by the level of the external K^+, until this concentration reaches 50 m.eq./liter. Even at this value the amount of K^+ entering the cell is only a fraction of that to be expected if the K^+ accumulation were due to a Donnan mechanism.

The above results have been obtained either with normal or low Na Ringer, when the calculations are made on a wet weight basis. The normal Na–high K Ringer does not produce a change in cellular volume (so the wet weight and dry weight results are comparable). The low Na–high K solutions induce a swelling of the cells which does not amount to more than 17 per cent even when the external K^+ level is 50 m.eq./liter. This swelling is much less than that obtained by Boyle and Conway under similar conditions. When one allows for this swelling, *i.e.* considers the results on a dry weight basis, there is an actual entry of K^+ into the cell which amounts to 40 per cent of the original content at 50 m.eq./liter external K^+. When the muscles are equilibrated in normal Ringer prior to soaking in low Na–high K Ringer the swelling and the increase in internal K^+ are reduced.

We shall now consider the alterations in Na^+ resulting from alterations in the external K^+ level in the unequilibrated series. In normal Na–high K Ringer there has been no alteration in Na^+ content except at the highest K^+ level in the unequilibrated series. At this level the entry of Na^+ which normally takes place on soaking has been hindered. However, high K^+ does not cause a reduction in Na^+ level once it has entered, since there was no difference in internal Na^+ between control and treated, when the muscle had previously been equilibrated in normal Ringer.

When the muscles are placed in low Na–high K Ringer, the level of internal Na^+ falls, so that the ratio Na^+_{out}/Na^+_{in} is greater in these muscles than in controls soaked in normal Ringer.

A perusal of Tables VI and VII shows that the isolated cell maintains a ratio Na^+_{out}/Na^+_{in}, approximately equal to 3 over a great range of external Na^+ (45 to 650 m.eq./liter). This it does by either extruding or imbibing Na^+ as the situation demands. In the presence of low Na^+ (45 m.eq./liter) the muscle contracts normally, although when stimulated the A.P. is some-

what reduced in height (see below). However, at the highest levels of Na^+ the muscle becomes non-excitable and bunched up after 30 minutes, and the intracellular K^+ falls greatly, in some cases being extruded, until there is none in the fiber. In spite of this apparently severe damage to the cell the normal Na^+ ratio is maintained. This contrasts with the previous finding (27) that the extrusion of Na^+ into low Na Ringer is inhibited in the cold. In this case the cell has not been able to maintain the usual ratio of 3. The only other deviation from this general finding was mentioned in the previous section, where a ratio greater than 3 was found in muscles treated with low Na–high K Ringer. It should be pointed out that this constancy of Na^+ ratio is only found in soaked preparations. Wide fluctuations, both greater and less than this ratio, are found in the intact animal (26).

The main conclusion to be drawn from these results is that the cell is able to maintain its internal K^+ at an *absolute level* despite great fluctuations (0 to 50 m.eq./liter) of the external K^+. This is in contrast to the capacity of the cell to maintain an *absolute ratio* Na^+_{out}/Na^+_{in} in the face of large variations in the external Na^+.

It is of interest that the only change in intracellular potassium obtained by alteration of the external ionic pattern was due to very high external sodium. Also the only alteration in Na^+ ratio was produced by high K–low Na Ringer, whereas normal K–low Na Ringer had no effect on this ratio.

In an earlier paper (26) we have shown that there is no simple correlation between the internal K^+ and Na^+ content of the cell, and the R.P. and overshoot respectively. This non-correlation between ionic ratios and bioelectric potentials was demonstrated from a consideration of variations in the internal ionic constitution whilst the external environment was held constant. In this paper the effect of variations of the external ionic environment on the bioelectric potentials has been considered. Our results substantially confirm those of our earlier paper, that there is no simple physicochemical relationship or conception which can connect the ionic gradients and the bioelectric potentials.

We have studied the alteration in R.P. following raising or lowering of the external K^+ level, and have obtained results which essentially confirm those of other workers (16, 17, 22). However, when these findings are considered in conjunction with the ionic analyses which were carried out in parallel with the bioelectric studies, it is clear that they do not support the existing theories. It is commonly said that alteration in the external K level results in a series of values for the R.P. which, when plotted against the log of the external K^+ concentration, has a slope of 58, except at low values of external K^+. From this, it is assumed that the R.P. is a "potassium potential." That the linearity of the above relationship does not hold over that range of external K^+ which is compatible with the appearance of an action potential is ignored.

When the K^+ was reduced below the normal Ringer level (even to zero) there was not a theoretical elevation of the R.P. This disparity has commonly been ascribed to a leakage of K^+ from the cell, which reduces the ionic gradient (4). The results of our ionic analyses show that there is no greater loss of K^+ into a K-free Ringer than there is into normal Ringer (29). Thus, in a K-free Ringer (the preparation having reached a steady state) the R.P. should be infinite, whereas the highest potential we have ever measured under this condition was 112 mv.

As the external K^+ concentration is raised there is still no marked alteration in the level of the R.P., the value for which only begins to decrease with a slope of 58 when the external K^+ is raised about fivefold. At this point there is a marked diminution in the ability of the muscle to produce an action potential on stimulation. When the K^+ is further increased, the muscle fails to produce any electrical response to stimulation. Although at this point the R.P. is falling off with the predicted slope the absolute values do not agree with those predicted by the Nernst equation, in fact the observed values are always more than 35 per cent higher than the calculated. If one calculates the K^+_{in} on a "fiber-water" basis the two values approach one another more closely but are still significantly different at the 1 per cent level. (See Table IV.) In agreement with results mentioned earlier in this paper the analyses of the individual muscles whose fibers had been penetrated showed that no K^+ had entered the cells except at the very highest levels of external K^+ (50 m.eq./liter).

The R.P. of muscles soaked in high K Ringer were similar, whether the excess K^+ was added to normal Ringer or whether an equivalent amount of Na^+ was replaced. Nor were the results affected by equilibration of the muscles in normal Ringer prior to immersion in the high K^+ solution.

In one respect our results with high K^+ solution were of particular interest. Muscles immediately immersed in Ringer containing 50 m.eq./liter K^+ with an equivalent amount of Na^+ subtracted yielded R.P. slightly but significantly lower than those of the companion muscles placed in high KCl–normal Na Ringer. Analyses showed that the only difference between the two muscles was that the former contained much less Na^+, and consequently the ratio Na^+_{out}/Na^+_{in} was increased (Table V). Whether the small drop in R.P. found between these series is of the order of magnitude predicted by the Goldman equation must await further work, including in particular the estimation of Cl^-. It must be noted that there is a marked difference in Cl^- content between the two Ringer solutions.

We have shown previously (26) that R.P. measured in the intact animal did not show any concomitant variation with the ratio Na^+_{out}/Na^+_{in}, although the value of this ratio varied from infinity to 1:2.

In distinction to the resting potential, the action potential is considered

to be a "sodium potential." The evidence for this assumption has again been provided by a study of the effect of alteration of the external Na^+, and it has always been assumed that the internal Na^+ remained constant.

The results of our potential measurements in general agree with those obtained by other authors. But the results of our analyses carried out on the penetrated muscles lead us to a different interpretation. We have previously pointed out that the level of the internal K^+ remains almost constant despite large fluctuations of the K^+ environment. Such is not the case with Na^+ for, when the level of the external Na^+ is altered, the ratio Na^+_{out}/Na^+_{in} remains constant at a value of about 3. Therefore other workers have been in error in assuming that the internal level was unaltered. Accordingly there should be no alterations in the magnitude of the overshoot with variation in the external Na^+. In agreement with other authors we find an immediate decrease in the overshoot when a muscle is placed in low Na Ringer, although this drop is not as great as that predicted by theory. As the Na^+ ratio returns to normal during the next hour there is no alteration in the size of the overshoot although a return to normal as the Na^+ ratio increases might be expected. It is noteworthy that Fatt and Katz (8) were able with crustacean muscle to obtain large values of the overshoot in solutions in which Na^+ had been replaced by other cations. The result of increasing the external Na^+ has been said to lead to an increase in the magnitude of the overshoot. The relative increase in external Na^+ was, however, small (125 per cent).

We have not observed an increase in A.P. in any of the high Na^+ series, neither in the mean value obtained during the first 10 minutes, nor in isolated potentials measured during this period. Work with radioactive Na^+ has suggested that the rate of diffusion of the ion through the muscle is much greater than the rate of exchange across the membrane (12, 18). Consequently, one would expect that soon after the muscle had been placed in high Na Ringer there would have been a transient increase in Na^+ gradient. This was never reflected in high A.P.

Exposure of the muscle to Na^+ levels of 400 to 500 per cent caused damage to the fibers. Normal R.P. and A.P. were obtained for up to 30 minutes, but after this time both potentials fell off rapidly and the tissue became unexcitable.

At the present time, it would appear that the majority view is that the resting and action potentials are due to the ionic gradients of K^+ and Na^+ respectively. These gradients are maintained by the active pumping out of Na^+ ions. The K^+ gradient is passively set by the activity of the Na^+ pump. More recently doubt has been cast on the above theory (10, 11, 25, 26, 29). Parlin and Eyring (23) have proposed a more radical theory that the resting potential results from the activity of the Na^+ pump. Grundfest (10, 11) also leans toward this view. We do not propose in this paper to put forward any hypothesis to account for either the bioelectric potentials or the maintenance

of the ionic gradients. This will be discussed in a later paper on the basis of results at present under review. We should, however, like to stress that the interpretation of our results is completely at variance with the majority view expressed above. The theory of Eyring is, by its nature, less easily checked by physical measurements, but it would seem difficult to reconcile with our previous finding (26) that the ratio of Na^+_{out}/Na^+_{in} varies from infinity to 1:2 in the intact animal, while the resting potential and internal K^+ remain essentially constant.

SUMMARY

The resting and action potentials of sartorius muscles of the toad, *Bufo marinus*, have been measured under varying conditions of external environment. At the same time, analyses for Na^+ and K^+ content were carried out.

There was a slight elevation of 2 mv. when the measurements were made in phosphate-Ringer instead of in bicarbonate-Ringer. The R.P. was independent of the hydrogen ion concentration between pH 6.5 and 8.5, although at these pH's there was marked alteration in the level of Na^+ and K^+ in the muscle.

Alteration of the external K^+ level between 0 and 50 m.eq./liter has little influence on the internal K^+ concentration. When the log of the external K^+ concentration is plotted against the R.P. there is not a linear relationship until the external K^+ is raised above 12 m.eq./liter, at which point the cell is unexcitable. Above this value a straight line with a slope of 58 mv. per tenfold change in concentration is obtained, but the absolute values at any point are about 35 per cent higher than those which would be given by the Nernst equation.

Alteration of the external Na^+ level within a range of 45 to 650 m.eq./liter resulted in marked changes in the internal Na^+ content, without, however, having any effect on the ratio Na^+_{out}/Na^+_{in}. This ratio has remained at about 3 in spite of marked fluctuations in the absolute value of the internal and external Na^+ levels. When the Na^+ level is lowered there is a decrease in the height of the action potential although there is no alteration in the ratio Na^+_{out}/Na^+_{in}. As the Na^+ level is raised the height of the action potential is not affected even in the presence of a fivefold increase in Na^+ in the Ringer.

The results do not support the conclusion that the bioelectric potentials can be calculated from the ionic ratios by means of simple physical chemical hypotheses such as the Nernst or Goldman equations.

The maintenance of the normal K^+ content of the cell cannot be accounted for by a Donnan mechanism. No definite evidence has been produced to explain the mechanism of a Na^+ "pump." In other words, the concept of a Na^+ pump requires that there shall be a physico- or organochemical mechanism which will distinguish between Na^+ and K^+ (or other) ions. There is evidence that Na^+ can be extruded against a concentration gradient. On the other hand

the cell is able to maintain a constant ratio of external to internal Na^+ even when the cell has been severely damaged by very high external Na^+ levels.

This work was carried out with the aid of a grant from the National Health and Medical Research Council of Australia. Mr. B. M. Johnstone was the recipient of a Burroughs Wellcome (Australia) Fellowship. We wish to thank Mr. George Stecher and Mrs. Suzanne Bennett for their skilled technical assistance. Our thanks are also due to Professor M. Belz and Mr. R. T. Leslie for their advice on some of the statistical procedures.

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OXYGEN PROTECTION OF BACTERIOPHAGE T1 AGAINST IONIZING RADIATIONS*

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(Received for publication, May 8, 1956)

Since the first unequivocal report of oxygen protection of biological material against ionizing radiations (6) the authors have extended the investigation in an attempt to elucidate the mechanisms involved. The question was raised in the initial report whether bacteriophage T1, the organism showing this phenomenon, was unique in this respect, since current literature shows that oxygen enhances radiation damage in most biological systems. The knowledge that oxygen is able to protect bacteriophage against ionizing radiations has evoked a special interest in the mechanisms operative. This is especially true since the results obtained with irradiation of the relatively simple, non-metabolizing phage system may make possible a more accurate estimate of the relative toxicity of the products of irradiated water in biological systems than could be obtained in the presence of more elaborate and complex systems.

An even greater interest in the work stems from the fact that Alper (3) in her earlier work with oxygen and phage S13 stated, "It was found that, during X-irradiation of oxygen-free phage suspensions, inactivation proceeded initially at a rate which was certainly no slower than that observed in aerated suspensions. The rate of inactivation became greater in the aerated suspensions when reaction with hydrogen peroxide had begun to be perceptible." Alper later (*cf.* reference 4 for summary) indicated the conditions under which oxygen protects phage S13. The results with phage S13 are at variance in certain important aspects with our findings with phage T1, a report of which is given here in detail.

Materials and Methods

Gamma Irradiation.—The 300 curie cobalt-60 gamma source of the Radiation Chemistry Project at Notre Dame was used for irradiation of samples. The general installation and protective lead barriers have been described by Ghormley and Hochanadel (9). A heavy brass device was constructed to assure the precise centering of the cobalt source, by remote control, in relation to the sample to be irradiated.

* Research performed under Contract No. AT(11-1)-205 between the Atomic Energy Commission and the University of Notre Dame.

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The dose was determined by the oxidation of ferrous ammonium sulfate to ferric ammonium sulfate in 0.8 N H_2SO_4. Over the 2¼ years during which this research was carried out, the dose rate of the Co^{60} gamma source, for the particular position of the irradiated vials, fell from 4050 r/min. to 3040 r/min., in keeping with the Co^{60} half-life of 5.3 years. Calculation of the dose rate was based on a G value of 15.8 (12), which represents the number of ferrous ions converted to ferric ions per 100 electron volts absorbed by the solution. All irradiations were carried out at 25°C.

Irradiations were repeated a minimum of ten times, or until the mean value of the point was established with reasonable certitude. Table I indicates the range of fluctuation; the significance of the standard error, or standard deviation of the mean, is explained by Snedecor (17). It should be noted that the analyses are based on the values of separately prepared and irradiated samples, and not replicate platings or dilutions of the same sample. These latter yielded highly accurate results when based on an average plaque count per sample of 500 to 600, secured from 3 to 5 Petri plates prepared by the agar layer method (2).

Each irradiated sample was paralleled throughout with an unirradiated sample which was treated identically except for irradiation. The survival fraction (N/N_0) is the ratio of the number of active phage after irradiation (N) to the number of active phage in the unirradiated control (N_0). Immediately after irradiation, 2 ml. of 3 per cent Difco nutrient broth was added to both the irradiated and control samples. The concentrated broth reacts with any remaining harmful products in the irradiated medium which would tend further to decrease survival. Viruses in this condition have been found to be stable for periods of 10 days or more in so far as the number of survivors did not change when aliquots of such samples were assayed daily; nevertheless, assays were completed within 2 hours of completion of irradiation.

Ultraviolet Irradiation.—A low pressure, mercury vapor germicidal lamp, manufactured by General Electric Company, giving essentially monochromatic radiation at 2537 A, was used in the experiments. The 15 watt lamp was mounted in a ventilated cabinet with controlled temperature. A shutter above the irradiated sample permitted precise timing of exposures, eliminated the necessity of turning off the lamp during a series of experiments, and thereby assured a more uniform dose. All exposures were made at a distance of 63 cm. from the lamp in vycor No. 7910 silica glass tubes of 15 mm. diameter. The UV absorption coefficient of the glass was 0.18 $mm.^{-1}$; the average wall thickness 1.14 mm. The radiant flux density in air of the UV source at 63 cm. was 755 ergs $cm.^{-2}$ $sec.^{-1}$.

Phage Stock.—Bacteriophage T1, specific for *Escherichia coli*, strain B, was used in all experiments. *E. coli* was grown in a chemically defined medium which was a modification of that described by Adams (2) and contained the following ingredients per liter of distilled water: lactic acid, 9 ml.; Na_2HPO_4, 1.4 gm.; $MgSO_4$, 0.05 gm.; NH_4Cl, 1.0 gm.; KH_2PO_4, 1.5 gm. The pH was adjusted with NaOH to obtain a final value of 6.8–6.9 after the solution was autoclaved.

After growth at 25°C. to a concentration of approximately 10^8 cells per ml., the bacterial suspension was inoculated with T1. After the phage had been permitted approximately 10 hours for lysis of bacteria, the culture was filtered through a fine Mandler diatomaceous earth filter. Since, after several weeks, the phage stocks showed a gradual but consistent loss of response to protection by oxygen during ir-

radiation, in all experiments the phage was used within 4 weeks after filtration. Unless otherwise specified, the stock of phage was diluted to produce an ultimate concentration of 5×10^{-5} that of the original lysate. In this concentration the radiation effects with the various gases were more pronounced than in higher concentrations of the original lysate (Table II in Results and Analyses).

TABLE I

Statistical Analysis of Values in Fig. 1

Treatment before irradiation		Roentgens of gamma irradiation	No. of experiments	Mean survival	Standard error
Oxygen saturation at 0°C.	Line A	8,500	10	5.12×10^{-1}	0.39×10^{-1}
		17,000	10	2.50×10^{-1}	0.29×10^{-1}
		25,000	10	1.10×10^{-1}	0.09×10^{-1}
Oxygen saturation at 25°C.	Line B	8,500	15	4.49×10^{-1}	0.06×10^{-1}
		17,000	15	2.25×10^{-1}	0.20×10^{-1}
		25,000	10	8.36×10^{-2}	2.10×10^{-2}
$O_2 + H_2$ saturation at 0°C.	Line C	8,500	10	2.54×10^{-1}	0.31×10^{-1}
		17,000	11	1.35×10^{-1}	0.22×10^{-1}
		25,000	10	6.01×10^{-2}	0.48×10^{-2}
$O_2 + H_2$ saturation at 25°C.	Line D	8,500	10	1.98×10^{-1}	0.29×10^{-1}
		17,000	11	9.99×10^{-2}	0.93×10^{-2}
		25,000	11	4.27×10^{-2}	0.48×10^{-2}
Equilibrated with air at 25°C.	Line E	8,500	10	1.30×10^{-1}	0.28×10^{-1}
		17,000	10	5.00×10^{-2}	1.05×10^{-2}
		25,000	10	1.66×10^{-2}	0.04×10^{-2}
$O_2 + CO$ saturation at 25°C.	Line F	8,500	10	6.57×10^{-2}	0.84×10^{-2}
		17,000	10	2.25×10^{-2}	0.74×10^{-2}
		25,000	10	6.62×10^{-3}	0.80×10^{-3}
Hydrogen saturation at 25°C.	Line G	8,500	10	3.20×10^{-2}	0.46×10^{-2}
		17,000	18	1.10×10^{-2}	0.24×10^{-2}
		25,000	10	3.92×10^{-3}	0.41×10^{-3}
Nitrogen saturation at 25°C.	Line H	8,500	10	4.01×10^{-2}	0.69×10^{-2}
		17,000	27	9.37×10^{-3}	1.50×10^{-3}
		25,000	16	3.33×10^{-3}	0.79×10^{-3}
CO saturation at 25°C.	Line I	8,500	13	2.05×10^{-2}	0.37×10^{-2}
		17,000	29	2.48×10^{-3}	0.53×10^{-3}
		25,000	12	1.91×10^{-4}	0.31×10^{-4}

Procedure with Gases.—Deoxygenation was accomplished by bubbling specially

purified nitrogen (99.9 per cent N_2) through the samples for 30 minutes. After this procedure there was no residual oxygen detectable by the Winkler test for dissolved oxygen, nor could the formation of any hydrogen peroxide after gamma irradiation of the deoxygenated samples be detected. The same method was employed for all gases or gaseous mixtures used.

Water was quadruply distilled: a preliminary distillation, successive distillations from alkaline potassium permanganate and alkaline manganous hydroxide, and distillation with no added solute. All glassware was pyrex; meticulous cleansing of glassware proved very important.

Once the proper concentration of phage was established, there remained the serious problem of rapid inactivation of the phage by bubbling of the gases. In appreciable concentrations of salts and/or protein, the phage is quite stable (1), whereas in distilled water the inactivation rate during bubbling is rapid and increases the fluctuations encountered in subsequent irradiations. This added instability was avoided by completing all gaseous bubbling before the phage was added. This was accomplished by utilizing pyrex test tubes, 15 mm. in diameter, which were sealed with B & D vacutainer stoppers through which stainless steel needles were introduced for bubbling and subsequently for introduction of the phage. The sealed ampoule was irradiated in an upright position with care that the phage suspension did not touch the vacutainer stopper during any of the manipulations.

Earlier work on the problem revealed that the results obtained in buffer differed from those obtained in distilled water. In order to assure complete equilibrium of phage and medium, the bacteriophage of the original lysate was diluted 100-fold, 24 hours before irradiation, in a medium identical to the one in which it was to be irradiated. In cases in which the phage was irradiated in gas-saturated solutions, this preliminary dilution was made in the proper gas-saturated solution 24 hours before irradiation. A final 200-fold dilution was made immediately before irradiation from these tubes into a freshly prepared medium, contained in sealed ampoules, by introducing a 10 lambda aliquot of diluted phage stock by means of an adapted micropipette into the 2 ml. sample after completion of the bubbling.

In those experiments concerned with varying pH by means of buffers, phage was diluted 100-fold in *distilled water* saturated with the proper gas, and 10 lambda of this diluted phage suspension was added to the experimental vial containing 2 ml. of the *buffer* 15 seconds before irradiation. This procedure was intended to maintain the effect due to the pH of the medium while permitting the minimal possible interaction of the salts at the viral surface.

In the series of experiments utilizing hydrogen peroxide, samples of water were bubbled with a particular gas and 10 lambda aliquots of hydrogen peroxide were subsequently introduced into the sealed ampoules by the same procedure used for the phage, to produce the proper concentration of hydrogen peroxide.

Chemical Analyses.—Oxygen content in micrograms per milliliter was determined by the Winkler method, adapted in this study to microdeterminations. All conditions under which the oxygen determinations were made paralleled those under which the viruses were irradiated. Hydrogen peroxide content was determined by the spectrophotometric method of Savage (15). Samples containing known quantities of hydrogen peroxide, to which the reagents were added at zero time and stored in the dark

at 0°C., were assayed spectrophotometrically 20 minutes after the addition of the reagents. A molecular extinction coefficient was determined from a standard curve relating optical density to known molarity of H_2O_2, according to the formula, $k = D/c \cdot l$, in which k is the extinction coefficient, D is the optical density as read on the Beckman model DU spectrophotometer, c is the concentration of hydrogen peroxide in moles/liter, and l is the light path in centimeters. The extinction coefficient under conditions outlined above was 22,200.

RESULTS AND ANALYSES

A phage stock grown in inorganic salts with lactate as the source of carbon and subsequently diluted in distilled water saturated with certain gases, constitutes a system very sensitive to ionizing radiations. Table II gives some pertinent information condensed from preliminary experiments, which may be briefly summarized as follows:—

1. Oxygen offers considerable protection from indirect effects of irradiation, as shown by the difference between survival in samples saturated with oxygen and with nitrogen. Nevertheless, oxygen permits considerable indirect action, as shown by the differences between survival in broth and in oxygen-saturated distilled water.

2. Oxygen protection shows a gradation dependent upon the amount of oxygen present.

3. The effect of diluting the original lysate from a concentration of 5×10^{-3} to 5×10^{-5} is considerable. In view of this finding, the greater stability of the system containing more lysate was sacrificed for the sake of obtaining results under conditions in which the minimal amount of lysate was present to alter the basic effects of irradiation. Except for certain values included in Table II, all results were obtained from experiments utilizing stocks diluted to a concentration of 5×10^{-5} before irradiation.

Survival of T1 Irradiated in Distilled Water Saturated with Various Gases.— In Fig. 1 are shown survival curves for T1, which verify the trend noted in the preliminary work that survival of T1 shows a relationship to the amount of oxygen present. Survival of T1 irradiated in distilled water which had been saturated with oxygen at 0°C. was higher than in distilled water which had been saturated with oxygen at 25°C., as expected from gas solubilities. Likewise, the survival values in distilled water saturated with oxygen bubbled simultaneously with hydrogen or carbon monoxide, at the same pressure as the oxygen, are higher than the values obtained by saturation with hydrogen or carbon monoxide alone, but lower than when saturated with oxygen alone. The intermediate values for the gaseous mixtures indicate that protection proceeds in so far as the water can be saturated with oxygen while a second gas is being bubbled into it simultaneously. It will be shown that hydrogen and nitrogen serve merely to deoxygenate the water, while carbon monoxide

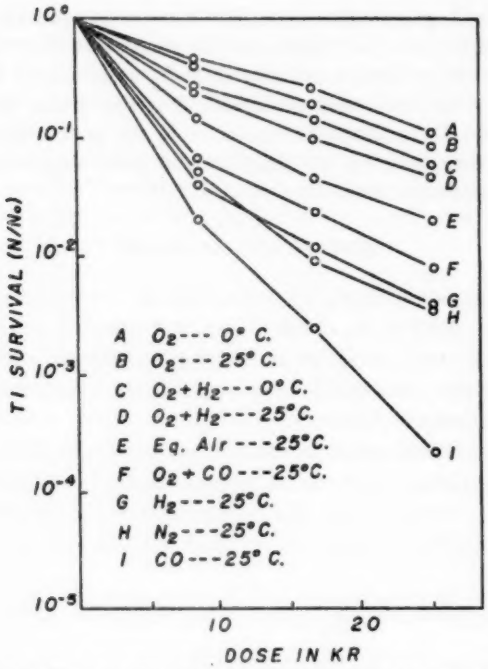

FIG. 1. Survival of T1 irradiated in distilled water which had been saturated at either 0°C. or 25°C. with various gases as indicated.

TABLE II

Survival of T1 after 45,000 r of Gamma Rays When Irradiated in Distilled Water through Which Oxygen in Varying Concentrations Had Been Bubbled for 30 Minutes

Dilution of original lysate	Gaseous treatment	Suspension medium	T1 survival N/N_0
5×10^3	None	Broth	7.25×10^{-1}
5×10^3	O_2 alone	Distilled water	1.13×10^{-1}
5×10^5	O_2 alone	Distilled water	1.25×10^{-3}
5×10^3	Equilibrated with air (20 per cent oxygen)	Distilled water	8.02×10^{-3}
5×10^3	90 per cent N_2; 10 per cent O_2	Distilled water	3.23×10^{-3}
5×10^3	95 per cent N_2; 5 per cent O_2	Distilled water	1.60×10^{-3}
5×10^3	N_2 alone	Distilled water	3.12×10^{-5}

has a somewhat deleterious effect. Carbon monoxide causes approximately 50 per cent inactivation of bacteriophage in 1 hour in unirradiated controls. The low survival of T1 irradiated in carbon monoxide indicates that in addition to the deleterious effect of carbon monoxide itself, the effect of gamma irradia-

tion is enhanced by its presence. In radiation chemistry the action of CO parallels the action of H_2, and any interpretation of its action must be made in the light of the results with H_2. The protective action of O_2, in the presence of CO, must also be interpreted in the light of subsequent analyses of H_2 results, with the toxicity of CO and the biological peculiarities of any test organism ever in mind.

Products of Irradiated Water.—Upon irradiation of water in the absence of oxygen (13), H and OH are produced according to the following equations:

$$H_2O \rightarrow H_2O^+ + e^- \quad (1)$$

$$e^- + H_2O \rightarrow H + OH^- \quad (2)$$

$$H_2O^+ \rightarrow H^+ + OH \quad (3)$$

$$H^+ + e^- \rightarrow H \quad (4)$$

The ionized water molecule formed in reaction 1 may recapture an electron to form an excited water molecule (14), which may then dissociate.

$$e^- + H_2O^+ \rightarrow H_2O^* \quad (5)$$

$$H_2O^* \rightarrow H + OH \quad (6)$$

In the presence of oxygen, HO_2 and H_2O_2 are produced.

$$H + O_2 \rightarrow HO_2 \quad (7)$$

$$2HO_2 \rightarrow O_2 + H_2O_2 \quad (8)$$

From Equations 1 through 8 it may be seen that there are four agents which are potential inactivators of bacteriophage: H, OH, HO_2, and H_2O_2. It is possible in the following experiments to assess the importance of these agents in the inactivation of phage T1, and, further, to determine the most probable means of inactivation. Under the following four headings the relative toxicity of these agents for T1 will be considered.

A. Atomic Hydrogen.—Since the survival of T1 is lower after irradiation in a suspension saturated with nitrogen than with oxygen, a deleterious agent which can exist at greater steady state concentration in the former than in the latter should be sought. This abundant agent is atomic hydrogen, which would logically be suspected as the agent responsible for phage inactivation. This point can be tested by saturating with hydrogen, in the absence of oxygen, the solution in which the viruses are irradiated. This procedure will favor the production of atomic hydrogen, according to the following equation:

$$H_2 + OH \rightarrow H_2O + H \quad (9)$$

The survivals of T1 irradiated in suspensions saturated with hydrogen alone and with nitrogen alone are not significantly different (Fig. 1). Radiation-produced hydrogen atoms alone are therefore inadequate to account for the

low survival in deoxygenated solutions, as demonstrated by this experiment. It does not follow that T1 is insensitive to H atoms; on the contrary, if T1 is sensitive to OH radicals, as will be shown later, then in these hydrogen experiments every H atom, produced at the expense of an OH radical, eliminates an agent known to be deleterious, and substitutes one which may be equally or somewhat less deleterious, in so far as this can be determined within the limits of experimental error.

T1 is rather susceptible to reducing compounds, as will be shown later when the protective effect of reducing compounds on irradiated T1 is considered. The fact that the hydrogen and nitrogen curves do not differ significantly may therefore be due to (*a*) the ability of the phage to compete successfully with H_2 for the OH radicals, or (*b*) a sensitivity to both H atoms and OH radicals, with some evidence of a greater sensitivity to OH radicals. It should be noted that (*a*) and (*b*) are not mutually exclusive, nor are other possibilities beyond the scope of our present knowledge excluded.

B. Hydroxyl Radicals.—From the above considerations it appears that the protection afforded by oxygen against gamma irradiation in aqueous suspensions cannot be attributed to the attractive hypothesis that oxygen competes successfully with the phage for the hydrogen atoms. It is important at this juncture to know whether phage is susceptible to OH radicals. This can be tested by reaction of the phage with free radicals produced in solutions of hydrogen peroxide subjected to ultraviolet irradiation. The reaction can be explained (11) as a primary dissociation into hydroxyl radicals, followed by reaction of peroxide with the hydroxyl radicals to give perhydroxyl radicals which react with each other to re-form peroxide, according to the following equations:

$$H_2O_2 \xrightarrow{uv} 2OH \tag{10}$$

$$OH + H_2O_2 \rightarrow H_2O + HO_2 \tag{11}$$

$$2HO_2 \rightarrow H_2O_2 + O_2 \tag{12}$$

For this study it is necessary to determine (*a*) the degree of inactivation of T1 exposed to a solution of *hydrogen peroxide only*; (*b*) the degree of inactivation of a suspension of T1 exposed to *UV only*; and (*c*) the degree of inactivation of a suspension of T1 exposed to *hydrogen peroxide and to UV simultaneously*. Since the rate of inactivation in all three cases is essentially exponential, it is possible to calculate whether the combined effect of UV and H_2O_2 is equal to the separate effects of UV and H_2O_2, or whether it exceeds them. The latter was found to be the case. The relative effectiveness of each treatment and of the combined treatment is shown in Table III, in which e^{-x} represents the survival fraction and x represents the dose in arbitrary units, responsible for the inactivation in each case. It is called "effective dose" in Table III, since

it represents the relative deleterious effect in each treatment. It represents the numerical value of D/D_1 in the equation $N/N_0 = e^{-D/D_1}$, in which N/N_0 is the survival fraction at dose D, and D_1 is the dose required to give a survival fraction of 0.368 or e^{-1}.

The effective dose under the combined treatment is greater than the sum of the effective doses when the two are operating separately. The typical example given above has been verified at other UV doses and hydrogen peroxide concentrations. Since T1 is inactivated more efficiently when exposed to hydrogen peroxide and UV simultaneously than when exposed separately, it is concluded that phage T1 is sensitive to OH radicals and probably to HO_2 radicals which may be formed concomitantly. Since both OH and HO_2 radicals are produced in the experiments involving UV, this test alone does not distinguish between the effects of the two. In the light of the experiments involving hydrogen and nitrogen, however, it is presumed that phage is susceptible to OH radicals,

TABLE III

Inactivation of T1 by UV and by Hydrogen Peroxide Acting Separately and Acting Simultaneously in Deoxygenated Solutions

90 Per cent confidence intervals are indicated.

Treatment	T1 survival e^{-x}	Effective dose x
H_2O_2 alone	$2.21 \times 10^{-1} \pm 4.9 \times 10^{-2}$	1.51 (1.31–1.76)
UV alone	$1.62 \times 10^{-2} \pm 6.0 \times 10^{-3}$	4.12 (3.81–4.60)
H_2O_2 during UV	$1.11 \times 10^{-3} \pm 5.4 \times 10^{-4}$	6.83 (6.41–7.47)

because in the samples deoxygenated with hydrogen, HO_2 production is negligible; with nitrogen, HO_2 may be formed, but the yield is low. HO_2 remains to be considered.

Perhydroxyl Radicals.—No direct test applicable to the phage can at present be devised to test the toxicity of HO_2. When HO_2 is produced in the various experiments outlined in this report, it is always produced together with other inactivating agents and its action cannot be isolated (*cf.* Equations 7, 11, 15, and 16). Certain considerations, however, indicate that HO_2 is probably an effective inactivating agent for T1. Since OH is harmful to phage and the action of HO_2 as an oxidant parallels that of OH, and since oxidizing agents will be shown to be of importance, it might be expected that phage will be sensitive to HO_2 radicals. At present, however, no clear-cut experimental demonstration of the sensitivity of T1 can be made.

D. Hydrogen Peroxide.—If one were to consider only the experiments utilizing samples deoxygenated by nitrogen and samples containing various amounts of oxygen, a progressive increase in the amount of hydrogen peroxide,

paralleling the increase in oxygen content, would be noted, together with the increased phage survival. This increase in phage survival which accompanied the increase in hydrogen peroxide might appear to be due to the reaction of the H_2O_2 with the OH and H of the irradiated water, indicated in reactions 11 and 13, but further experiments disprove this hypothesis.

$$H_2O_2 + H \rightarrow H_2O + OH \tag{13}$$

It is possible to test this point by a manipulation whereby the oxygen content can be *decreased* and the hydrogen peroxide content *increased*. By bubbling hydrogen and oxygen through the solutions simultaneously, a lower oxygen

TABLE IV

Relation of Oxygen Content and Hydrogen Peroxide Production to Survival of T1 after 17,000 r of Gamma Irradiation

Survival is shown in relation to oxygen assays made before irradiation and to hydrogen peroxide assays made after irradiation. Vials used for chemical assays were handled in every respect like those in which T1 was irradiated. Each value is based on a mean of ten experiments.

Temperature during bubbling	Gas or gases	O_2 content	H_2O_2 content	T1 survival N/N_0
°C.		μg./ml.	μg./ml.	
0	O_2	37.8	1.08	2.50×10^{-1}
25	O_2	30.3	1.02	2.25×10^{-1}
0	$O_2 + H_2$	28.3	1.23	1.35×10^{-1}
25	$O_2 + H_2$	21.2	1.17	9.99×10^{-2}
25	Equilibrated with air	7.88	0.84	5.02×10^{-2}

content was obtained, but a higher hydrogen peroxide yield was secured upon irradiation according to Equations 9, 7, 12, and 14, in that order.

$$HO_2 + H \rightarrow H_2O_2 \tag{14}$$

The higher hydrogen peroxide production in the presence of hydrogen, with lower oxygen content, has been verified in our experiments (Table IV, Figs. 2 and 3). It is most significant, however, that the survival of phage is directly correlated with the oxygen content and not correlated with H_2O_2 production.

The fact that hydrogen peroxide can react to remove certain radicals produced by irradiation of water, as illustrated in Equations 11 and 13, has led to the suggestion that hydrogen peroxide added to the medium in which the phage is irradiated might serve as a protective agent. A series of experiments, therefore, was designed to check the ability of H_2O_2 to protect phage under various conditions. Fig. 4 shows the result of irradiating bacteriophage in the presence of 2.5×10^{-4} N H_2O_2 saturated with various gases. In general, a decreased survival of T1 results when hydrogen peroxide is present in the suspen-

sion. Survival is increased in the presence of oxygen and lowered in the presence of hydrogen or nitrogen. Under the conditions of these experiments, therefore, hydrogen peroxide added to the suspension of phage before irradiation not only fails to protect but actually increases the inactivation.

It has been proposed (4) that the inactivation of phage by hydrogen peroxide is due to the reducing action of free radicals. The mechanism whereby

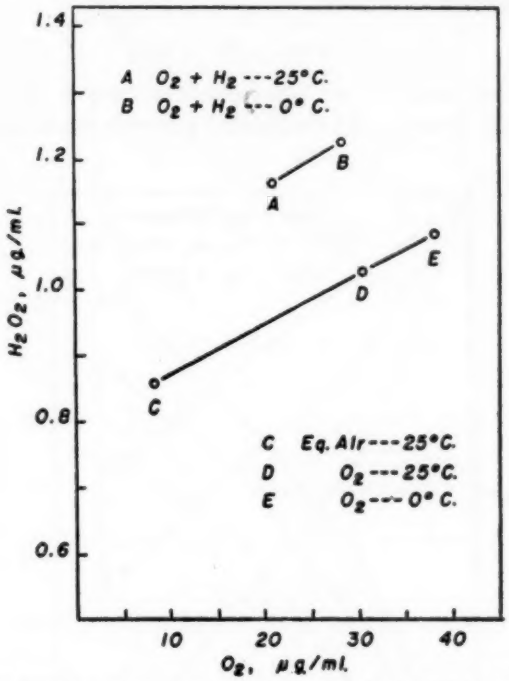

FIG. 2. Effect of 20 kr. of gamma rays in production of H_2O_2 expressed in micrograms per milliliter and plotted as a function of oxygen content at beginning of irradiation. Samples contained O_2 alone, or mixtures of H_2 and O_2.

H_2O_2 inactivates bacteriophage is of great importance in this study. H_2O_2 can act as an oxidizing agent or as a reducing agent. In the following equations T1 represents the undamaged phage, while $T1^+$ represents the oxidized phage and $T1^-$ the reduced phage.

$$T1 + H_2O_2 \rightarrow T1^+ + OH + OH^- \quad (15)$$

$$T1 + H_2O_2 \rightarrow T1^- + H^+ + HO_2 \quad (16)$$

The mechanisms whereby the radicals, HO_2 and OH, formed in the decomposition of H_2O_2, or during irradiation, may inactivate the phage are as

follows:—HO_2 may inactivate the phage by either oxidation or reduction. Oxidation proceeds according to the following equation:

$$T1 + HO_2 + H^+ \rightarrow T1^+ + H_2O_2 \quad (17)$$

Reduction proceeds according to the following equation:

$$T1 + HO_2 \rightarrow T1^- + H^+ + O_2 \quad (18)$$

which may be written as follows:—

$$T1 + O_2^- \rightarrow T1^- + O_2 \quad (19)$$

These last three reactions are pH-dependent, because of the equilibrium $HO_2 \rightleftharpoons H^+ + O_2^-$. Reaction 17 (oxidation) proceeds more readily in an acid

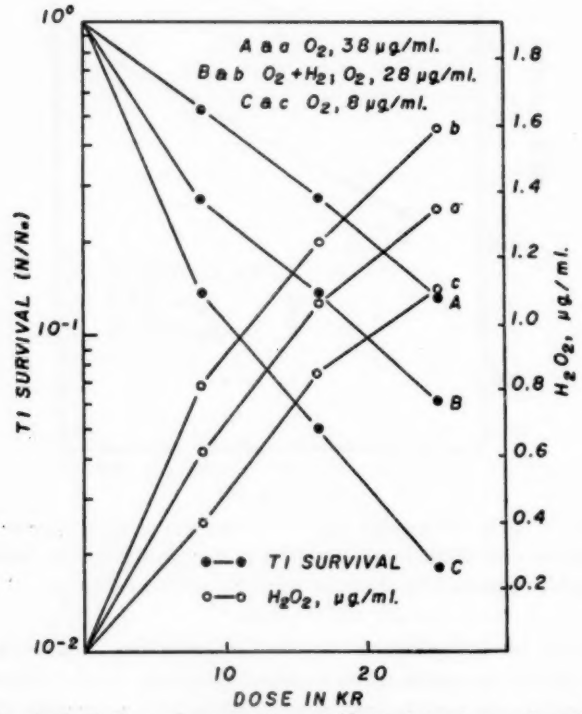

FIG. 3. Survival of T1 and production of H_2O_2 plotted as a function of dose of gamma rays. T1 was suspended in distilled water which contained a known quantity of O_2 at beginning of irradiation; H_2O_2 was assayed at end of irradiation.

medium. Reactions 18 and 19 (reduction) are favored by an alkaline medium. Since inactivation of the phage proceeds more readily in an acid medium and the phage is more stable in an alkaline medium during irradiation, it ap-

pears that oxidation rather than reduction is the more important mechanism of inactivation for phage T1.

This conclusion is strengthened by the following considerations of the action of the OH radical. From the equilibrium, $OH \rightleftharpoons O^- + H^+$, suggested by Hart

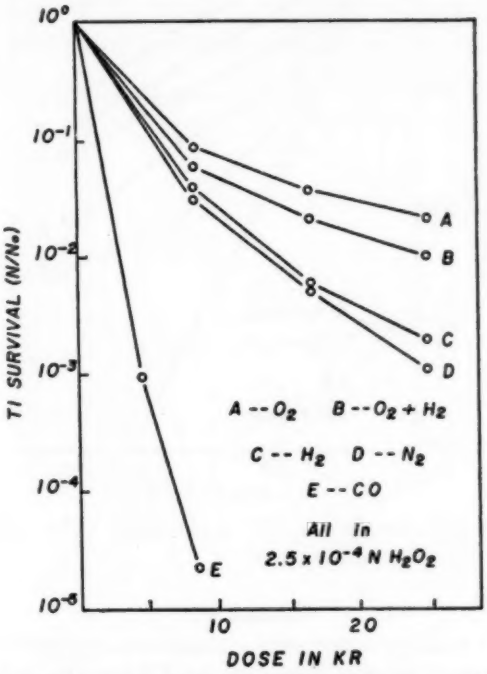

FIG. 4. Survival of T1 irradiated in 2.5×10^{-4} N H_2O_2 saturated at 25°C. with various gases, as indicated.

TABLE V

T1 Survival (N/N_0) after Standing 5, 10, and 15 Minutes in 10^{-3} N Hydrogen Peroxide Saturated with Oxygen, Hydrogen, and Nitrogen, Respectively

Gas	5 min.	10 min.	15 min.
Oxygen	6.25×10^{-1}	3.92×10^{-1}	2.45×10^{-1}
Hydrogen	3.50×10^{-1}	1.30×10^{-1}	4.32×10^{-2}
Nitrogen	3.30×10^{-1}	1.20×10^{-1}	3.68×10^{-2}

et al. (10), we deduce that in an alkaline medium OH radicals react as follows: $OH + OH^- \rightarrow O^- + H_2O$. Since O^- is not as efficient an oxidizing agent as OH (8) and since phage is sensitive to OH, this explains why T1 is inactivated more readily in an acid than in an alkaline medium during ir-

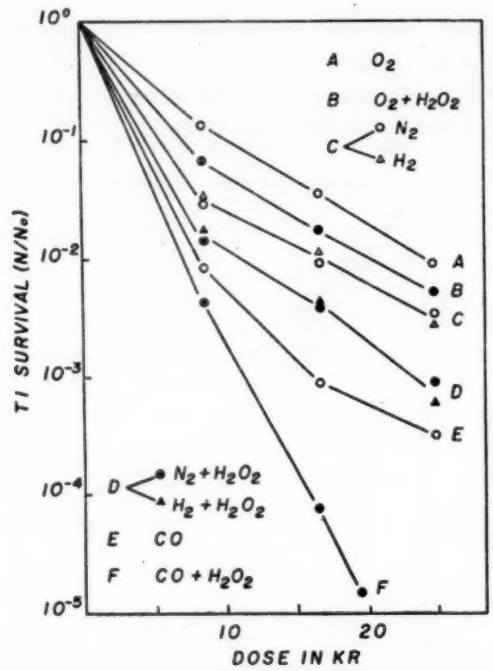

FIG. 5. Survival of T1 irradiated in 10^{-2} M phosphate buffer adjusted to pH 6.75 and saturated at 25°C. with various gases.

TABLE VI

Survival of T1 Irradiated with 17,000 r of Gamma Rays in Solutions Containing Known Amounts of Oxygen in Micrograms Per Milliliter: One Series in 10^{-3} M Phosphate Buffer, One in Distilled Water

No. of experiments	Temperature during bubbling	Medium	O_2 in	T1 survival N/N_0
	°C.		μg./ml.	
10	25	Buffer + O_2	30.0	2.12×10^{-2}
10	25	Buffer + O_2 + H_2	18.5	1.06×10^{-2}
10	25	*d.* H_2O + O_2	30.3	2.25×10^{-1}
10	25	*d.* H_2O + O_2 + H_2	21.2	9.99×10^{-2}

radiation. The higher inactivation rate in an acid medium suggests, therefore, the importance of the oxidizing action of the OH radical.

It is not evident how oxygen may enter into any of these reactions in the role of a protective agent, yet oxygen does protect the phage against inactivation in solutions of H_2O_2 (Table V) as well as during irradiation (Fig. 4). There is no adequate explanation at present for this phenomenon, but we sug-

gest that oxygen may function in the role of protective agent for T1 by the formation of a complex with the phage. Two possibilities are suggested: either the phage reacts with oxygen prior to irradiation to achieve this protection, or, in the very process of inactivation, the oxygen forms a stabilizing complex with the phage, as will be considered in the Discussion.

Influence of Buffer in the Phage-Gas System.—Although the advantages of working with T1 in distilled water have been discussed, for the sake of complete-

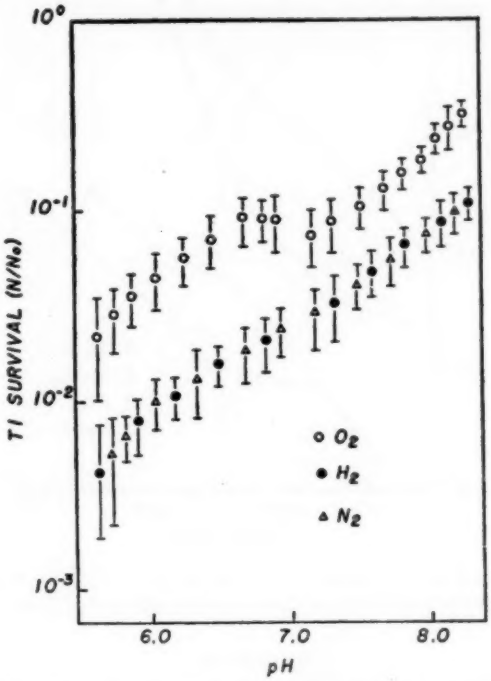

FIG. 6. Effect of pH on the survival of T1 irradiated with 13,500 r of gamma rays in 10^{-3} M phosphate buffer which had been saturated with O_2, H_2, or N_2 at 25°C. 95 per cent confidence intervals are indicated.

ness the corresponding pattern in phosphate buffer has been worked out. From a comparison of Fig. 5 with the preceding figures, it can be seen that the pattern is essentially similar, with one important exception: the protection afforded T1 by oxygen in distilled water is considerably higher than the protection afforded in buffer. It is here proposed that adsorption sites saturated with ions of the buffer solution may not be free to react with the oxygen present in the medium. The oxygen content and the corresponding survivals are shown in Table VI.

Hydrogen Ion Concentration.—In view of the importance of pH in deter-

mining the mechanism of inactivation of viruses by irradiation, as indicated above, a series of experiments was undertaken to establish the effect of pH on the phage system under irradiation. Fig. 6 shows that 10^{-3} M phosphate buffer

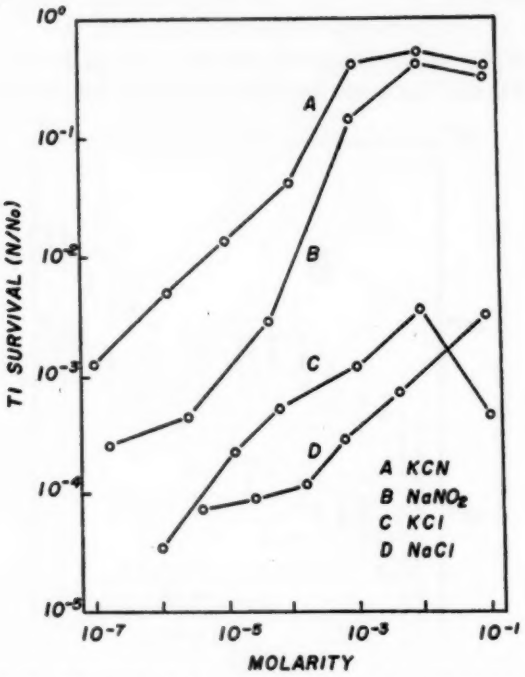

FIG. 7. Survival of bacteriophage T1 x-irradiated in various molarities of reducing agents (KCN, $NaNO_2$) and non-reducing agents (KCl, NaCl) at a dose of 37,500 r.

TABLE VII

T1 Survival after Standing in Reducing Compounds for Designated Periods (No Irradiation)

Compound	T1 survival 3.5 min.	Compound	T1 survival	
			3.5 min.	15 min.
$NaNO_2$ saturated	4.8×10^{-1}	KCN 0.1 M	1.6×10^{-1}	8.9×10^{-2}
$NaNO_2$ 1 M	7.7×10^{-1}	KCN 0.01 M	8.6×10^{-1}	7.4×10^{-1}
$NaNO_2$ 0.1 M	9.0×10^{-1}	KCN 0.001 M	9.7×10^{-1}	8.8×10^{-1}
$NaNO_2$ 0.01 M	9.9×10^{-1}	KCN 0.0001 M	1.0×10^{0}	9.0×10^{-1}

saturated with oxygen, hydrogen, or nitrogen, gives higher survivals in the alkaline range. From extensive experimentation in this laboratory there has come a consistent pattern of increased radiation sensitivity at the higher

hydrogen ion concentrations. This pattern has been found to hold in radiation experiments involving sulhydryl compounds (unpublished), inorganic salts (7), organic acids and their salts (5), as well as the present work with phosphate buffer.

Protection of Phage by Reducing Agents.—The implications of pH in determining radiation sensitivity have led us to conclude that oxidation rather than reduction is the more important factor in the inactivation of T1 in the present

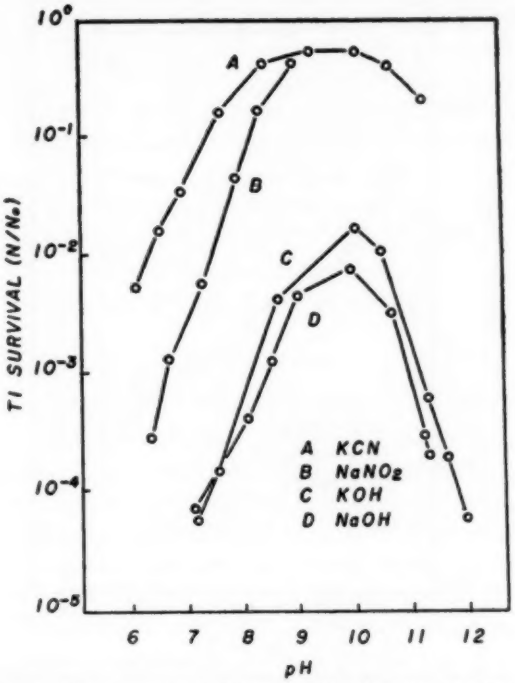

FIG. 8. Effect of pH on survival of T1 irradiated with 37,500 r of x-rays while suspended in KCN, $NaNO_2$, KOH, and NaOH.

study. In view of the opposite interpretation with S13 (4), a careful study of this point has been made with a number of reducing agents. For the sake of brevity only the results obtained with sodium nitrite and potassium cyanide are given; these lend themselves readily to experimental manipulation. It is recognized that the nitrite ion can be either oxidized or reduced, but the redox potentials of irradiated water are such that nitrite is oxidized by irradiation. The reducing agent appears to divert to itself some of the oxidizing power of the irradiated water which, in the absence of the reducing agent, would appear as molecular H_2O_2 (16), or would inactivate the phage. In accordance with the free radical theory, Schwarz and Allen (16) suggest that OH radicals,

formed in fairly close proximity to one another, which would have an appreciable probability of combining to form H_2O_2 in the absence of the reducing agent, react with the reducing agent when it is present.

Although the phage showed a certain sensitivity to these compounds, as indicated in Table VII, the significant point in this study is that the susceptibility to reduction is slight compared to the sensitivity to indirect effects of

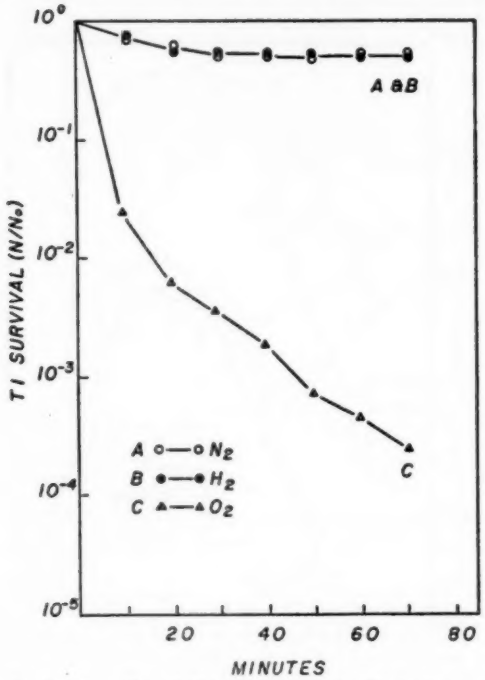

FIG. 9. Survival of unirradiated T1 added to distilled water saturated at 25°C. with O_2, N_2, or H_2 and irradiated with 37,500 r of gamma rays. Survival (N/N_0) represents ratio of active phage at given standing time to active phage at zero time.

x-rays or gamma rays, against which these reducing compounds are very efficient protective agents. This protective effect and its magnitude can be seen in Fig. 7, in which KCN and $NaNO_2$ show considerable protection over NaCl and KCl; the latter are non-reducing under conditions of these experiments and show minimal protection among selected inorganic salts (7).

The range of pH for the concentrations of salts shown in Fig. 7 was: KCN, 6.4 to 11.5; $NaNO_2$, 6.3 to 8.5; NaCl, 5.4 to 6.7; KCl, 6.2 to 6.8. In view of the consistently higher survivals as the pH increased to approximately 10, it was necessary to adjust the pH of the solutions of KCN and $NaNO_2$ in order to distinguish between the protective effects of basicity and of reduction. In Fig. 8 are shown the results of varying the pH by varying the concentration

of the salt, as contrasted with the results obtained with the use of NaOH and KOH. The differences between the curves for KCN and $NaNO_2$ and their corresponding bases represent the protection attributable to the specific action of CN^- and NO_2^-; namely, reduction. The greater sensitivity of T1 to irradiation in the acid range with these salts substantiates the observations made above relative to hydrogen ion concentration.

Postirradiation Effects.—The survival of T1 was consistently less when it was added to irradiated water which was saturated with oxygen than when it was added to irradiated water saturated with nitrogen or hydrogen (Fig. 9). This sharp contrast to the protective effect of oxygen during irradiation may be explained as follows: the chief products of irradiated deoxygenated water are OH radicals and H atoms. Since the half-life of these products is extremely short—in terms of fractions of microseconds—it is necessary that the phage be present *during* the irradiation in order that any appreciable effect be shown. In oxygen-saturated solutions, however, H_2O_2 persists, and the phage is sensitive to H_2O_2. All traces of protective agents, *e.g.* protein, must be eliminated from the solutions to bring out these effects.

DISCUSSION

The fact that oxygen protects bacteriophage T1 against ionizing radiations whereas it sensitizes most biological systems suggests immediately that T1 may be basically different from these other systems and may, in fact, be inactivated by reduction rather than by oxidation. The extensive test of this hypothesis here reported, however, indicates that these viruses are fundamentally similar to other systems in that they are inactivated chiefly by the oxidizing action of ionizing radiations. This is of great importance in the interpretation of radiation effects, and gives significance to effects obtained with viruses in other studies which might otherwise be misleading. The fact that oxygen does protect the phage, however, remains to be explained. It appears that oxygen functions in a manner not at the present time completely amenable to the conventional analyses of radiation chemists. From the evidence presented, it is proposed that oxygen functions by reacting in some manner with the phage particle itself to form a stabilizing complex. This complex may be formed before the actual events of inactivation by irradiation take place. In this case the binding of O_2 at sensitive sites would protect these sites against damage by the products of the irradiated medium. The complex may be formed, on the other hand, during the actual inactivation process and may be the result of such reactions as

$$OH + T1\text{-}H \rightarrow H_2O + T1\cdot \quad (20)$$

or,

$$H + T1\text{-}H \rightarrow H_2 + T1\cdot \quad (21)$$

then,

$$T1\cdot + O_2 \rightarrow TIO_2 \quad (22)$$

In the above reactions it is suggested that an OH radical or H atom may remove an H atom (or other group) from the phage, which process does not produce inactivation immediately. Before the phage "radical" (T1·) can become inactivated, an oxygen molecule stabilizes it. In favor of this second hypothesis of complex formation over the first is the fact that if there is a stabilizing union between the phage and oxygen *before* the beginning of the inactivation process, how does it happen that this reaction of phage and oxygen can be so readily reversed by merely bubbling nitrogen through the same phage suspension? The second hypothesis circumvents this difficulty. The mechanisms suggested in reactions 20 to 22 would, of course, be applicable to the protection of T1 against inactivation by the radicals of H_2O_2.

Although ultimately all of the inactivation by indirect effects of ionizing radiations is due to the production of free radicals and peroxides and their reaction with the phage particle, it would be misleading and even erroneous to presume that the phage particle itself is an accurate and unfailing indicator of effects exclusively at the chemical level. A consideration of other possible modifications of this biological entity, beyond those which can be accounted for purely on the basis of radiation chemistry, is imperative. This point is emphasized by the observation that cultures of phage stored at 4°C., in which the titer remained constant, showed a gradual but progressive failure with time to respond to the oxygen protection against irradiation observed with these same cultures initially. Obviously no genetic factor or maturation could be operative in a sterile stock of stored phage. The observation of the decline in oxygen protection with age of phage, as well as the diminished protective effect of oxygen in cases in which T1 has stood in buffer, both suggest that certain complexities of the phage particle itself must be taken into consideration before it can give an adequate picture of reactions in radiation chemistry.

The authors are grateful to Doctor Milton Burton of the Radiation Chemistry Project at Notre Dame for encouragement and suggestions in the early part of the work and for making available the cobalt – 60 gamma source; to Doctor Harold Dewhurst of the General Electric Company Research Laboratory, Schenectady, for many valuable discussions while he was at Notre Dame and for reading and criticizing the manuscript; to Doctor C. J. Hochanadel of Oak Ridge National Laboratory for valuable suggestions and for checking the manuscript.

SUMMARY

Bacteriophage T1 was suspended in distilled water and in phosphate buffer, saturated with oxygen, nitrogen, hydrogen, and carbon monoxide, and irradiated with gamma rays and x-rays. Under the same conditions phage was exposed to hydrogen peroxide.

Oxygen acted as a protective agent against both irradiation and hydrogen peroxide inactivation. As a protective agent against irradiation, oxygen was

more efficient in distilled water than in buffer. The phage was much more sensitive to irradiation in the presence of hydrogen or nitrogen than in the presence of oxygen. Survivals of phage irradiated in suspensions saturated with hydrogen and with nitrogen did not differ significantly. From this it was concluded that oxygen did not protect T1 by removing atomic hydrogen from the irradiated medium, since the hydrogen-saturated medium increased the yield of atomic hydrogen but did not increase the yield of inactivated phage. It was presumed, therefore, that phage is sensitive to OH radicals and this was confirmed by irradiating phage with UV in the presence of hydrogen peroxide and comparing this survival with the survivals obtained from hydrogen peroxide alone and from UV alone. The combined effect of hydrogen peroxide and UV acting simultaneously was greater than the effect attributable to hydrogen peroxide and UV acting separately.

Evidence for sensitivity to HO_2 radicals was considered, and the effect was attributed chiefly to an oxidizing action since phage sensitivity is greater at higher hydrogen ion concentrations, which favor oxidation by HO_2 radicals.

Since the OH radical is a more efficient oxidizing agent than O^-, the former being favored in an acid medium, the latter in an alkaline medium, and since the phage is more sensitive in the first situation than in the second, the present tests proved the importance of oxidation as the mechanism of inactivation. Since some inactivation was encountered when phage was exposed to reducing agents, independently of irradiation, it was concluded that phage is somewhat sensitive to reducing agents, but the inactivation attributable to ionizing radiations is due chiefly to oxidation, against which these reducing agents are very efficient protectors.

Under no circumstances did hydrogen peroxide protect T1, whether produced by irradiation in the medium or added beforehand to the medium to be irradiated. The first point was investigated by irradiating T1 in the presence of hydrogen and oxygen combined; this produced a higher yield of hydrogen peroxide but a lower survival of T1. In all these tests phage survival under irradiation was directly correlated with oxygen content of the medium rather than with production of hydrogen peroxide. It is proposed that the protective effect of oxygen is due to a reaction between the phage and oxygen, and this complex confers stability upon the phage.

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Note added to Proof.—In our current research the protection by oxygen has been established at dose rates as low as 1/10 the rate reported in this paper; this gives additional support to the conclusions here reported.

EVIDENCE FOR THERMAL REACTIONS FOLLOWING EXPOSURE OF DIDINIUM TO INTERMITTENT ULTRAVIOLET RADIATIONS*

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(Received for publication, May 31, 1956)

Many previous workers have found low intensity ultraviolet (UV) radiation less effective than high intensity UV in producing injury to various living organisms (Christensen, 1953; Coblentz and Fulton, 1924; Dreyer, 1903; Gates, 1929; Swann and del Rosario, 1932; Weinstein, 1930; Wyckoff, 1932). Experiments performed here on the protozoan *Didinium nasutum*, indicated an opposite, greater effect of low intensity UV. It appeared likely that a dark reaction succeeds the absorption of UV, so that quanta are supplied at high intensity more rapidly than they can be utilized; *i.e.*, saturation occurs. To investigate this possibility the UV was flashed and the dark period between flashes varied, with the expectation of increased injury with longer dark periods. Since a dark reaction is thermochemical, it should proceed more rapidly at high than at low temperature, and injury should therefore increase with temperature. Experiments were accordingly carried out at various temperatures. The expectations have been confirmed in the present study. The effects of variable light period and flash rate, and the comparison of flashed high intensity with continuous low intensity irradiation have also been explored.

Materials and Methods

Cultures of *Didinium nasutum* were grown in 4 mm. bore isolation tubes on concentrated suspensions of *Paramecium caudatum* in the manner previously described (Brandt *et al.*, 1955). Didinia selected at random for a sample were irradiated in a quartz cell, put into isolation tubes with food, one animal per tube, and the number in each tube was recorded three times daily, as a rule, until the fourth division was reached in all tubes. This stage was usually reached earlier in some tubes than in others, and the tubes were discarded as they attained it, since higher counts were difficult to make, and were likely to be biased because of rapid depletion of the food supply.

* Supported in part by contract AT(11-1)234 with the Atomic Energy Commission, in part, particularly the analysis of data, by Public Health Contract C-1799(C2).

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When observations for divisions had to be made for more than 48 hours, the didinia were gently centrifuged to the bottom of the isolation tubes, the supernatant fluid was removed under a dissecting microscope (× 6) with a mouth pipette, and a fresh suspension of paramecia was added. This is necessary because didinia deteriorate if fed on starved paramecia (Beers, 1926). All counts and observations were made in red light to avoid the possibility of photoreversal.

Intensity was varied by reducing the maximum intensity with one or more fine copper screens placed between the radiation source and the first lens of the monochromator. Each intensity was measured with the thermopile. Filters of cellophane were tried at first, but their fluorescence was a source of visible light, which introduced a considerable error in the determination of the intensity of UV, and also may have produced some photoreversal of the UV effects.

For most experiments UV of 2654 A from a quartz monochromator was used and the intensity was determined with a calibrated thermopile. Tests were made with dosages of 2000, 3000, and 4000 ergs/mm.^{2}/sec. and the dosage chosen in a particular experiment depended upon the tests to be made. For example, if long dark periods were used the largest dosage induced such a marked delay in division that it was not convenient for routine experimentation, although it gave some revealing information; a lower dosage would therefore be employed. Experiments with monochromatic UV were performed at room temperature (21–24°C.), but the didinia were grown at 27°C. in a dark incubator.

When the effect of temperature was being tested, a Westinghouse sterilamp which gives off about 85 per cent of its radiation at 2537 A was employed because it was impossible to bring the monochromator and ancillary equipment into the constant temperature rooms in which the experiments had to be performed.

It was not possible to obtain an accurate absolute measure of the intensity of the UV from the sterilamp under the conditions of operation, but measurement with a Hanovia UV meter made it possible to demonstrate that the intensity was constant during the period of experimentation; therefore the dosages were the same from experiment to experiment. A biological effect (the delay to the fourth division) was used as a criterion to set the initial dosage for the temperature experiments. This dosage or some fraction of it was then used in all subsequent experiments.

Temperature experiments were performed in constant temperature rooms at 5, 13, 25, and 30°C. All equipment, pipettes, and solutions were brought to the constant temperature room several hours before treatment and the didinia were brought in an hour before the experiment, since preliminary testing showed this time to be adequate for them to reach the temperature of the room. After an experiment the didinia were transferred to isolation tubes and put in the 27°C. incubator in the usual manner (see Brandt *et al.*, 1955). They were therefore exposed to the lower or higher temperatures only during the preexperimental incubation period and during irradiation, and for a short time before being transferred.

Flashing was performed by rotating a black paper disc with sectors cut out of it in front of the slit leading to the reaction cell. The disc was attached to a motor by a pulley. The speed of flashing was set at the start of an experiment with a strobotac.

In most experiments a sample of 10 didinia was taken for each test and for each control; smaller samples were occasionally necessary, and samples of 11 or 12 were

used at times. Data for a sample thus consisted of a series of counts obtained from each of about 10 tubes.

The first step in the statistical reduction of the data has been the computation, from each sample, of the mean time to the fourth division ($\bar{T}_4$), and the standard deviation (σ) of this time. The time to the fourth division in a given tube when the final number of animals was exactly 16 was taken as the time from isolation to that count; and when more than 16, was determined by interpolation between last and next to last counts, using a chart with logarithm of number of animals plotted against time. In a few instances, when the final count was less than 16, it was necessary to extrapolate from the last two counts.

In comparing results, $\bar{T}_4$ was always calculated using these conventions, and confidence limits for $\bar{T}_4$ were determined, or tests of significance of the difference between means made, using tables of Student's distribution (Dixon and Massey, 1951).

This procedure is subject to the general objection that only a fraction of the information obtained from each sample is used in the computed statistics. For this reason, special pains have been taken to study the data before its analysis, with a view to detection, follow-up, and reporting of any significant departures from normal patterns.

In many samples, one or more specimens failed to reach the fourth division. The procedure followed in such instances has been to compute mean and variance of the remainder of the sample, and to record the number of mortalities so as not to be misled by the statistics. The risk of bias is small when the sample is one with slight injury or none, for the dead specimens are then so far out of line with the rest of the sample that they may safely be regarded as gross errors, or as members of a different population (injured in manipulation, or contaminated, for instance). Occasionally a specimen which reached the fourth division, but much later than all others in the sample, would be excluded from the computation for the same reason, and with almost equally small risk. But the risk of bias is much greater when this procedure has to be followed in a sample with severe injury, for the dead specimens are then much less likely to represent gross errors, and should by rights be included. Yet they have an infinite T_4, and there is hence no choice unless the sample is thrown out entirely. Since valuable information would often be lost if the sample were thrown out, it was deemed the lesser of evils to follow the regular procedure, carefully noting mortalities and drawing no inferences without taking them into account.

Experimental Results

1. Nature of UV Injury.—The experiments performed here provide examples of UV injury which run the gamut from none (as in dark controls) to death before the first division in all specimens of a sample. The pattern of increase of numbers in a sample is not a simple exponential pattern, except in dark controls, and is even quite complicated in instances of severe injury. To report only $\bar{T}_4$ and its standard error is to report none of this complexity, and this is to exclude from consideration some features of general interest for the light which they throw on the nature of UV injury; moreover such procedure risks giving a false impression of simplicity of the raw data and adequacy of the

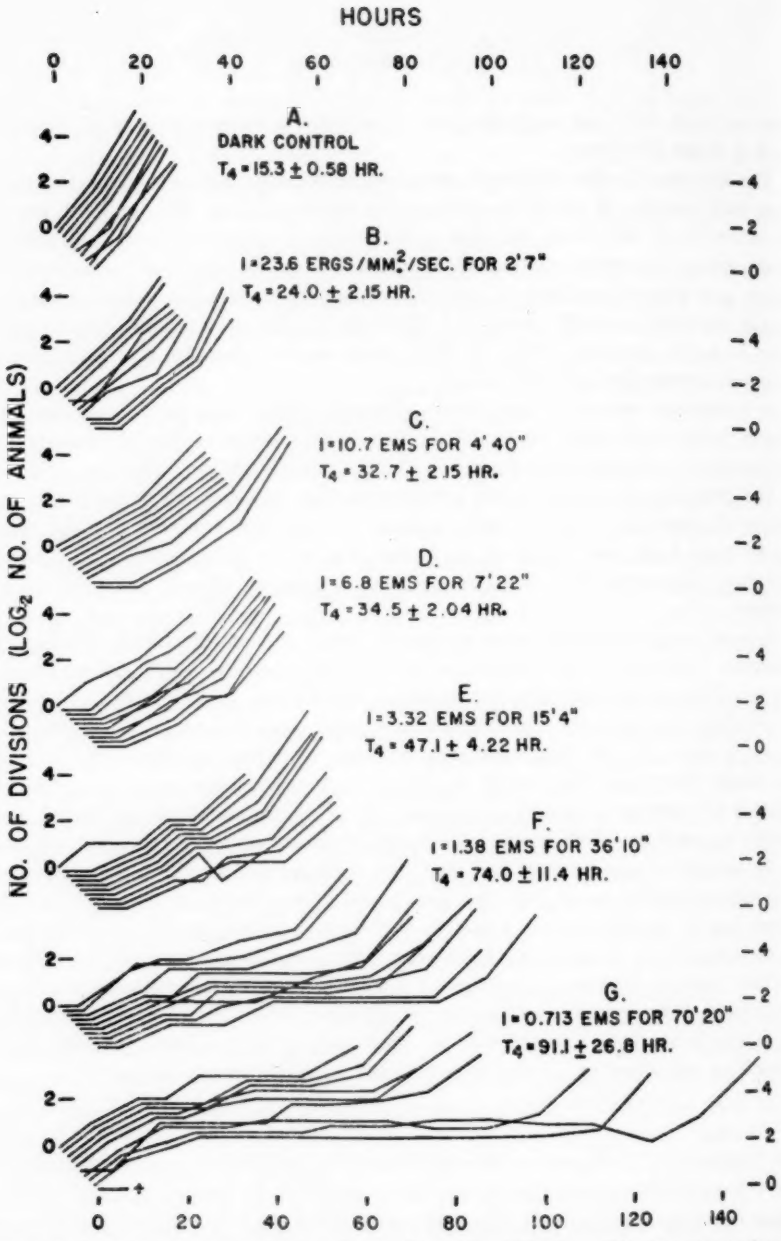

FIG. 1. Pattern of division delay induced by ultraviolet: graphs of a typical series of experiments, with dosage of 3000 ergs/mm.² at λ2654 A. The intensities decrease consecutively down the page (I = 23.6 to 0.713 ergs/mm.²/sec.). T_4 is the time to the fourth division after irradiation (or inoculation, in the dark control); it is represented by mean ($\overline{T}_4$) ± standard deviation (standard error of individual specimen). The curves are staggered for clarity, with two sets of coordinates given: one set at the top and left for the upper specimen in each sample; the other at bottom and right for the lower specimen in each sample. By interpolating between the given scales, the number of animals counted at a particular time can be accurately determined for any intermediate specimen.

statistics computed from them. A proper appraisal is facilitated by Fig. 1, in which representative samples are illustrated in such a way as to include all data from each specimen (tube), and to permit any given specimen to be followed through the entire course of its growth, from isolation through the fourth division.

The figure reveals the typical patterns of injury which have been found in scores of experiments with *Didinium*. Slight injury results in an immediate "slowdown" of division, with gradual and continuous recovery, *i.e.* the first division after irradiation is delayed longest and the normal rate is more closely approached with each subsequent division, being usually restored in full by the fourth division after irradiation.[1] This is the pattern for a short exposure at high intensity (Fig. 1 B). As intensity is lowered and exposure increased in duration to maintain a constant dosage, the slowdown increases somewhat (Fig. 1 C), depending on wave length.

But the greater injury which occurs with still lower intensity and longer exposure appears not as further slowdown, but as a "stasis" after the second division (occasionally after the first or third).[1] If the injury is not fatal, normal division rate is restored by about the fourth division, after a time in stasis which varies from a few hours to at least 100 hours, depending on the degree of injury (Figs. 1 D, E, F, G).

It will be noted that the more severely injured samples show much more variability. This increase of variability, which is reflected in the increase in standard deviation of T_4, appears to be attributable fundamentally to greater variability in time of stasis. Two specimens in the most severely injured sample (Fig. 1 G) show a decline in numbers before ultimate recovery (also one in Fig. 1 E, but this is most likely because of a mistake in counting), which obviously contributes to the larger variability of that sample; yet it is clear that the major contribution is from the variability of time in stasis.

2. The "Intensity Effect."—The increase of injury with decrease of intensity is evident in Fig. 1, which has been compiled from various experiments in which a dose of 3000 ergs/mm.^{2} was given at λ2654 A. Approximately 95 per cent confidence limits for $\bar{T}_4$ have been extracted from the data of Fig. 1 and plotted in Fig. 2, along with similar results from experiments with the same dose given at three other wave lengths.

Indications are that there is no intensity effect at intermediate and high intensities (6 to 36 ergs/mm.^{2}; 2654 A is exceptional). Injury rises rather abruptly at lower intensities, however, down to the lowest values which could be administered within the limits of accuracy of calibration of the arc. It is to be expected that at still lower intensities a peak of injury would be reached

[1] These characteristics were also found by Kimball, Geckler, and Gaither (1952) for *Paramecium aurelia*, except that injury was increased by raising the dose instead of lowering the intensity.

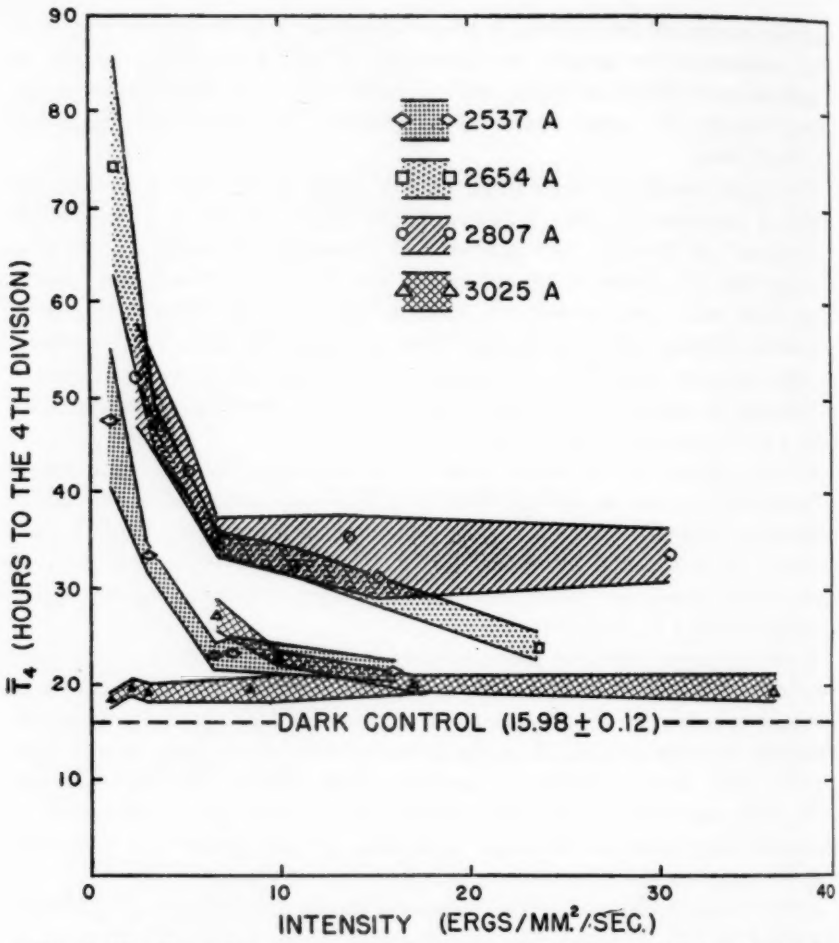

FIG. 2. The "intensity effect." Time to the fourth division of didinia after irradiation by each of four wave lengths of UV with the same dosage (3000 ergs/mm.²/sec.) delivered at different intensities. The points are the mean values of T_4 and the width of the bands indicates 95 per cent confidence limits at the points. At λ3025 A one series of experiments (April 24, 1954) suggests a slightly greater effect of low intensity than of high; a second more extensive series (September 24, 1955) performed at lower intensities does not; a third series (not illustrated) in which little injury occurred also showed no increase in effect at low intensities.

and passed, since repair of UV injury is probably occurring even during irradiation, or because the number of quanta of UV falling in unit time becomes insufficient to injure the cells. Unfortunately it was not practicable to investigate these lower intensities.

The occurrence of the confidence bands of the various wave lengths in pairs at different levels suggests an action spectrum of UV effect on division in *Didinium* with a maximum at the intermediate wave lengths. No systematic investigation of the UV action spectrum has yet been reported for division delay in *Didinium*, but the results provided by these experiments are in general accord with those obtained from more direct and extensive work on *Paramecium* and other forms (Giese, 1945; Kimball, 1955).

In the λ2537 and λ3025 A curves the level of $\overline{T}_4$ at high intensities is so near that of the dark control that nothing of interest is to be expected at still higher intensities. The $\overline{T}_4$ "plateau" at λ2807 A is at a much higher level, however, and it would be desirable to know the behavior of this curve as intensity is further increased. It would likewise be advisable to follow the λ2654 A curve to higher intensities. The high values obtainable with λ3025 A were not obtainable with the other wave lengths, however, because their spectral lines are much weaker.

Experiments performed to extend the range of intensities to lower values at λ3025 A have had the unexpected result that no intensity effect was shown at this wave length, perhaps because injury is not marked (Fig. 2). This phenomenon calls for further investigation, which it has not yet been feasible to undertake here. It suggests that the UV responsible for the "intensity effect" may have a rather narrow action spectrum in the range of short waves, from perhaps λ2500 to λ2800 A.

3. Intermittent Light.—If UV of intermediate and high intensity is less effective than UV of low intensity because the energy of quanta absorbed is not utilized in subsequent thermal reactions, flashing the light should increase its efficiency, as it allows time for the thermal reactions.[2] The data bear out this expectation, as can be seen by comparing results of continuous irradiation with those of flashed UV in Figs. 3 and 4, or in Fig. 5.

In the expectation that increasing the dark period would increase the injury, experiments were run using the largest possible range of dark periods within experimental limits. It proved to be impossible to obtain results over the whole range at a given dose and temperature; if any injury was to be obtained with the shorter dark periods, there would be too much with the longer ones, and the investigation of the longer dark periods was therefore accomplished by lowering the dose or the temperature.

Results are shown in Table I for experiments performed at 25°C. with the sterilamp at an intensity of 37 ergs/mm.^{2}/sec. In Table I A there is a slight increase of $\overline{T}_4$ with dark periods from 0.0039 to 0.0246 sec., and then a marked rise at 0.0532 sec. This pattern is at variance with the asymptotic increase of

[2] Studies at room temperature by Christensen (1953) indicated that intermittent UV is more effective than the same dose of continuous UV on *Tetrahymena*, suggesting thermochemical reactions in this case.

effect to be expected of a thermochemical reaction following a photochemical reaction (*cf.* Emerson and Arnold, 1932), but it may be due to a non-linearity of $\bar{T}_4$ as a measure of injury, rather than to anomalous behavior of the reactions themselves. The rise of $\bar{T}_4$ is not so steep at the still longer dark periods, as shown in Table I B from experiments with a lower dose. It was expected that a maximum would be approached, and the results suggest this possibility, although they cannot be considered conclusive.

Since time for thermochemical reactions is gained equally well by irradiating at a slower rate, one might assume that if light is delivered to the cell in about

TABLE I

Increase of UV injury with length of dark period between flashes: results of experiments at 25°C. with the sterilamp at an intensity of 37 ergs/mm.²/sec. The "40/90" disc means that a 40° sector was cut out of each 90° quarter, etc. The various discs were rotated at speeds such that the duration of flashes (light period) was constant at 0.0031 sec. Results on specimens exposed to continuous UV are included for comparison under disc, "none."

Dose, *erg/mm.²*	Disc	Dark period	$\bar{T}_4 \pm \sigma$, *hrs.*	
			First experiment	Repeat experiment
		sec.		
A. 6000	None	0	24.4 ± 2.3	27.3 ± 3.5
	40/90	0.0039	26.9 ± 2.1	28.0 ± 2.8
	20/90	0.0109	30.8 ± 2.2	36.0 ± 8.6
	15/90	0.0156	38.4 ± 6.2	33.5 ± 3.6
	10/90	0.0246	35.9 ± 4.0	34.1 ± 6.4
	5/90	0.0532	86.0 ± 34.7	74.3 ± 10.1
B. 4000	None	0	18.0 ± 1.9	
	5/90	0.0532	26.3 ± 3.3	
	5/180	0.1096	37.7 ± 6.3	
	5/360	0.222	39.7 ± 4.0	

the same time but in one case the light is flashed and in the other it is delivered continuously at lower intensity, the efficiency of the two treatments should be about the same. There were many experiments performed which provided suitable bases for comparison, in that the dose and over-all time of exposure were nearly identical. Of over 200 such experiments, 142 revealed instances in which flashed light was somewhat more injurious than continuous, 65 in which the opposite was true, and a few were ties. The probability of obtaining by chance such a deviation from equal numbers in each category is one in ten million. There is thus a good basis for the inference that flashed low intensity UV is slightly more effective than continuous low intensity UV delivered at the same average rate, though it is not easy to see why this is so.

A number of experiments were performed using the same disc with a favor-

able constant ratio of light to dark periods but varying the rate of rotation and hence of flashing (in the most extensive experiment, 100 to 12,896 cycles/min.; light period, 0.0126 to 0.000098 sec.; dark period, 0.1370 to 0.0011 sec., respectively). The expectation was that an optimum flash rate might be found for a given light-dark ratio. The results have not borne this out, however. In some experiments differences significant from the statistical standpoint have appeared, but no over-all pattern is discernible. Thus in one experiment the slowest flash rate may produce most injury, in another the fastest, and in a third, an intermediate rate. Often all flash rates produce nearly identical results. The most reasonable assumption is therefore that significant differences, when they appear, are attributable to factors other than flash rate. For this reason the experiments are not documented here.

One experiment was performed with practically constant dark period and variable light period. The UV injury was found to increase with decreasing light period; thus the T_4 was 26.4 $\pm$ 3.0 hours for 0.00333 sec. and 51 $\pm$ 11.7 hours for 0.00083 sec., from which it can be concluded that the time of the light reaction is less than the shortest light period used (0.0008 sec.), but it may well be much shorter than this. Equipment was not available to test shorter light periods, however.

4. Temperature and Dark Period.—If there is a thermochemical or dark reaction between UV absorption and the manifestation of injury, as suggested by the intensity effect, it should be possible to increase injury by raising the temperature as well as by flashing the light. Also, long dark periods should be more effective than short ones, regardless of temperature. Experiments were therefore performed at several temperatures using the same series of dark periods at each temperature, with results which confirmed these expectations: for a given dark period, the injury increased with temperature, and at a given temperature, the injury increased with dark period.

Figs. 3 and 4 represent the results of comparable parts of these experiments in full detail, in the same way as in Fig. 1. Each group of 10 specimens represents a sample treated at one of four temperatures and one of two dark periods (light period was constant at 0.0031 sec.). The samples of the shorter dark period are plotted in Fig. 3, along with the continuous light controls; those of the longer dark period are plotted in Fig. 4. The increase of injury with temperature is evident in both series, and so is the increase of injury with dark period, except at 5°C., where as expected only slight injury occurred in either case.

In Fig. 5 the values of $\overline{T}_4$ computed from these data are plotted as a function of temperature, showing the increase of injury with temperature more directly. The $\overline{T}_4$ values from a series of controls of zero dark period are also plotted (lower curve in the figure); these manifest only very slight injury at all temperatures, and their increase of injury with temperature is of questionable significance, though the trend is upwards. The increase of injury with dark period is

shown also by the fact that the higher curve corresponds to the longer dark period.

The difficulty of statistical reduction of the raw data can be appreciated from the study of Figs. 3 and 4. It is indeed open to question whether the adopted

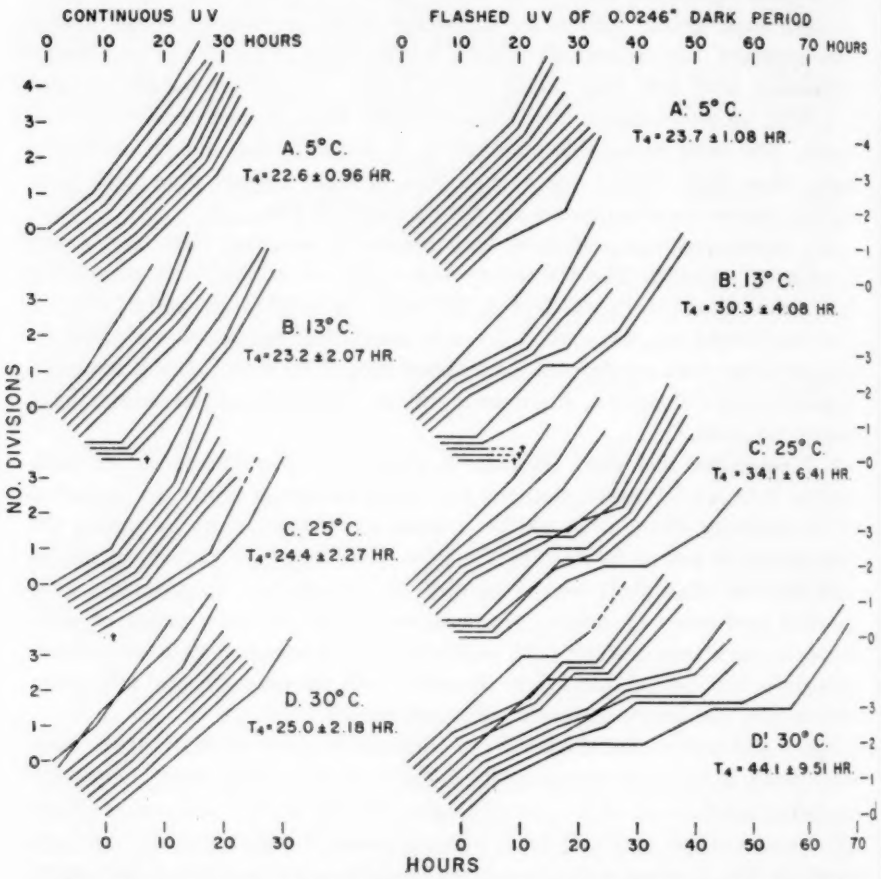
CONTINUOUS UV
FLASHED UV OF 0.0246" DARK PERIOD
HOURS
A. 5° C.
T4 = 22.6 ± 0.96 HR.
B. 13° C.
T4 = 23.2 ± 2.07 HR.
C. 25° C.
T4 = 24.4 ± 2.27 HR.
D. 30° C.
T4 = 25.0 ± 2.18 HR.
A'. 5° C.
T4 = 23.7 ± 1.08 HR.
B'. 13° C.
T4 = 30.3 ± 4.08 HR.
C'. 25° C.
T4 = 34.1 ± 6.41 HR.
D'. 30° C.
T4 = 44.1 ± 9.51 HR.
NO. DIVISIONS
HOURS

FIG. 3. Increase of UV injury with temperature, for continuous light and light flashed with dark period of 0.0246 sec. Results are plotted as in Fig. 1, with specimens going from highest to lowest in each sample in order of their speed of recovery, and with origins staggered both horizontally and vertically, to permit the clearest complete representation of the data.

measure of injury ($\overline{T}_4$) is adequate for the purpose. It is evident first of all, from the high degree of variability among the specimens of a sample, that $\overline{T}_4$ is subject to large error, particularly in those samples which show considerable injury. The standard errors of $\overline{T}_4$ were computed, and 95 per cent confidence

limits determined for each value by means of standard techniques (Dixon and Massey, 1951). There is general overlap of these limits at 5°C., but not at the higher temperatures. Thus statistical analysis confirms the unsophisticated impression from the raw data of significantly greater injury with longer dark

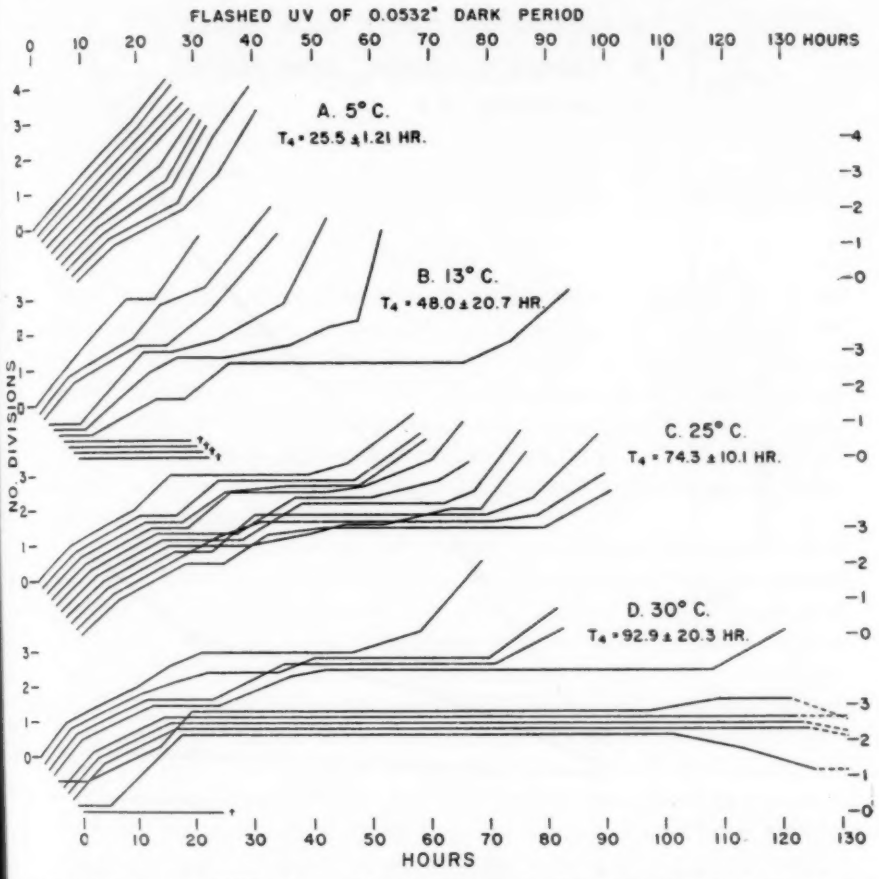

FIG. 4. Increase of UV injury with temperature for light flashed with dark period of 0.0532 sec. Figs. 3 and 4 both represent results of experiments with the sterilamp at an intensity of 37 ergs/mm.²/sec. Where the light was flashed, the light period was constant at 0.0031 sec. Time to the fourth division is given as mean ± standard deviation, as in Fig. 1.

period. The confidence limits of adjacent points at different temperatures on the same curve overlap in some cases, but those of alternate points do not, except the controls (lower curve), and there is therefore statistical support also for the impression of a real rise of injury with temperature.

There is doubt as to the validity of $\bar{T}_4$ and its standard error as a basis of comparison in a few instances. Specimens which never divided after irradiation were treated as gross errors in the sample, and therefore did not enter into the

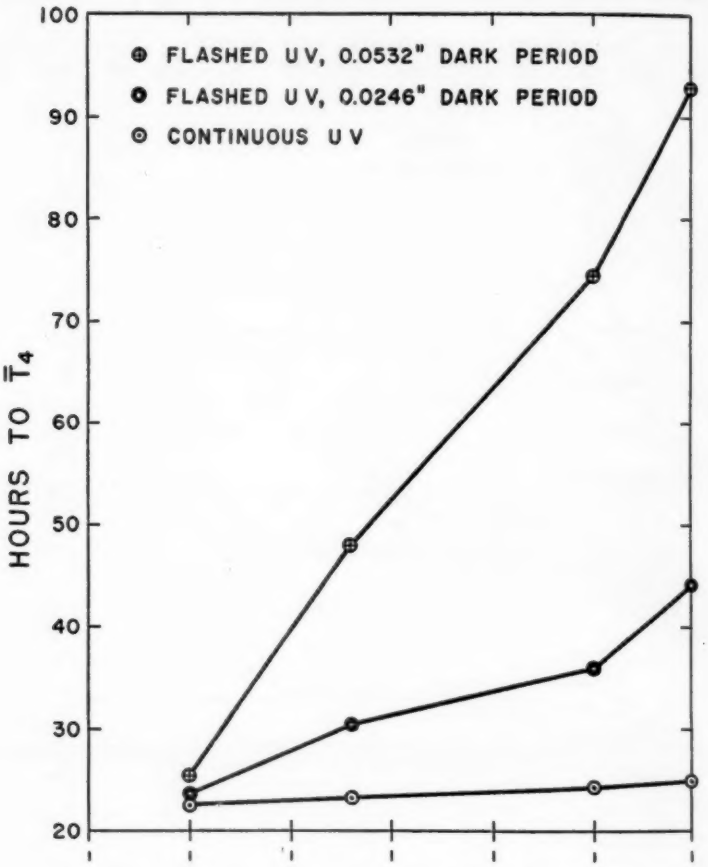

FIG. 5. Increase of UV injury with temperature, using values of T_4 extracted from the data of Figs. 3 and 4.

computation of $\bar{T}_4$. Since it has been our experience that specimens occasionally fail to divide even in non-irradiated controls, this procedure seems quite justified in the case of Fig. 3 B′. However, to have four failures in a control is rather rare; therefore, the procedure is subject to more doubt for Fig. 4 B. The exceptionally large variability of this sample may cast further doubt on its statistics. It is

clear, however, that the surviving specimens do show on the whole more injury than those at 5° and less than those at 25° of the same dark period, and also more than those at 13° of the shorter dark period.

It is a matter of certainty rather than of doubt that the statistics of Fig. 4 D are in error, since the five specimens which divided but which never reached the fourth division are hardly gross errors, The value of $\bar{T}_4$ is in this case unquestionably too low, since data for the infinite delay of the five specimens never reaching the fourth division are not included; but even so, it is the highest value among all the samples.

Thus there is nothing in the statistics to confute the conclusions which may be drawn by inspection of the data, that injury increases with temperature and dark period, and in fact they add some further support to these conclusions.

DISCUSSION

UV has three main effects upon the division pattern of didinia: (1) It decreases the rate of division so that the curve, plotting the logarithm of the number of individuals against time, declines in slope; (2) It induces a temporary cessation of division or stasis when the injury is marked, the length of the stasis depending upon the injury which in turn depends upon the dosage, the intensity, the temperature, the light period and the dark period; (3) It may kill the didinia if the stasis becomes so prolonged that recovery is impossible, or by inhibiting posttreatment division altogether.

At the present time no information is available to pinpoint the injuries which change the division pattern. It seems most likely that synthesis of some material essential for division (DNA?; see Kelner, 1953; Kimball, 1955) is blocked by UV and that so long as a store of this material is available divisions continue for a time after irradiation, but that stasis develops when this material has been consumed (Brandt *et al.*, data to be published). The retardation of the divisions preceding stasis might then be interpreted as resulting from injury of another sort, possibly to the cytoplasm, but in any case at a locus other than that producing stasis. That UV affects different components of the protozoan is demonstrated by the different types of action spectra observed for different effects on the same cell; for example, sensitization to heat approximates the absorption spectrum of cytoplasmic proteins, while division delay approximates that of nucleic acid (Giese, 1945).

The investigation of photosynthesis with flashing light (Emerson and Arnold, 1932) was based on the proposition that there was a single photochemical reaction which set the stage for a series of dark reactions. The dark reactions might actually be many and varied, and complexly interrelated, but their overall behavior would be like that of a single reaction which would go asymptotically toward completion at a speed that would vary with temperature and could be approximately determined experimentally with flashing light by increasing

the dark period between flashes until a maximum effect was obtained. The maximum rate of photosynthesis per unit dose of flashed light was shown to increase with temperature and to be approached asymptotically with increasing dark period, in accordance with this hypothesis.

UV injury to *Didinium* increases with temperature in a similar way. At a given temperature it also increases with dark period, but not asymptotically, as evidenced by the much greater steepness of the highest curve in Fig. 5. This may indicate a non-proportionality between degree of injury and amount of dark reaction, which offers difficulties of interpretation. It is more likely, however, that it is a reflection of the non-linearity of our measure of injury.

However that may be, it seems clear that thermochemical reactions follow in a very short period of time the absorption of UV by didinia. No reason exists to doubt that this conclusion is also applicable to other organisms.[2] It should therefore provide another tool for the analysis of UV effects on living matter.

SUMMARY

1. The nature of ultraviolet injury and its variation with the same dose given at different intensities and wave lengths have been investigated in the protozoan *Didinium nasutum*, using time to the fourth division as a measure of injury.

2. The injury has been found to consist of a "slowdown" of division rate, which always occurs, and a "stasis," usually at the second division after irradiation, which appears in varying degrees among more severely injured samples.

3. Injury was found to be almost independent of intensity at three wave lengths out of four studied over a wide range of intermediate and high intensities, but was found to rise sharply with lower intensity at all except the longest wave length.

4. Flashed UV of high intensity is much more effective than the same dose of continuous radiation at high intensity and shorter total time of treatment. It is also more effective than the same dose at low intensity and equal time of treatment, though only slightly so.

5. An increase of injury with rise of temperature and with increase of dark period clearly indicates that injury depends on thermochemical reactions following the absorption of UV in *Didinium*.

6. The most reasonable assumption is that a similar conclusion applies to other organisms as well, and that its general application may be useful in the investigation of UV effects on protoplasm.

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THE KINETICS OF CARDIAC GLYCOSIDE INHIBITION OF POTASSIUM TRANSPORT IN HUMAN ERYTHROCYTES*

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(Received for publication, April 26, 1956)

The present study of the kinetics of the inhibition of K transport in glycoside-poisoned human red cells has been undertaken in an effort to throw more light on the detailed mechanism by which K enters the normal cell. In 1953, Schatzmann (1) reported that cardiac glycosides inhibitied in a marked manner the uptake of K at 37°C. by red cells which had been depleted of intracellular K by cold storage. Since then Schatzmann's studies have been confirmed and extended by Joyce and Weatherall (2), Glynn (3), and Kahn and Acheson (4). The specific aspects of the problem that have been explored in the present study are: the development of the necessary kinetic treatment to cover the behavior of a heterogeneous three compartment system that is not in the steady state; the provision of a theory to account for the quantitative relationship between glycoside concentration and its effect on K transport; and a comparison of the glycoside–red cell dissociation constants with the mean lethal dose of the glycoside in the cat.

Experimental Method

The experiments on K influx into the red cell were carried out with fresh heparinized whole human blood incubated at 37°C. with K^{42} using the methods previously described in detail (5). A small amount of isotonic K^{42} Cl is added to the blood at the beginning of the experiment, and samples of plasma are taken for assay at hourly (or more frequent) intervals over the 4 to 5 hour course of the experiment. In a typical experiment, four aliquots of blood are incubated simultaneously, one serving as control, and each of the other three containing a different concentration of the same drug. In addition to the radioactive determinations in each plasma sample, plasma K was determined at the beginning, middle, and end of the experiment. Whole blood K, glucose, pH, and hemolysis were measured at the beginning and end of the experiment. In general, pH did not shift by more than 0.15 unit during the course of the experiment, and hemolysis was less than 0.3 per cent (maximum hemolysis 0.5 per cent).

* This research has been supported in part by the Atomic Energy Commission.

‡ Student Fellow of the United States Public Health Service during part of this research.

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Radioactivity measurements were made in a scintillation counter, and the counts were corrected for decay in the usual fashion. The other analyses were carried out using the techniques previously described in detail (5).

We are indebted to Dr. Otto Krayer, Dr. K. K. Chen, Sandoz Chemical Works, Inc., and Burroughs, Wellcome and Company for generous supplies of the drugs used. Ouabain was dissolved in isotonic saline in all experiments subsequent to M-4 (June 9, 1954); prior to that time, it was dissolved in water. The other drugs were dissolved in 95 per cent ethanol, since they are not sufficiently soluble in water. The same amount of solvent was added to the control as to the vessels containing the drug: in the case of ouabain up to 1 ml. of solvent to 40 ml. of blood; in the case of the other drugs, less than 0.1 ml. of solvent to 40 ml. of blood (because of the marked effect of ethanol on K flux (6)).

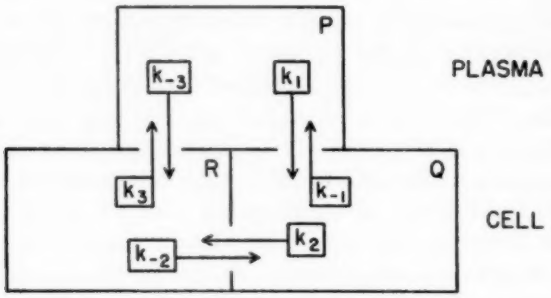

FIG. 1. Schematic representation of three compartment system for K transport in human blood.

Theoretical Model System for Potassium Transport

The results of previous experiments on K influx and efflux, as analyzed on an analogue computer, have been shown by Solomon and Gold (7) to lead to the choice of a three compartment symmetrical system as the simplest model that could be used to fit the data. As shown in Fig. 1, the extracellular phase was taken as constituting one compartment, P. The other two compartments, Q and R, were cellular, and it was suggested that the smaller which constitutes about 5 per cent of the total cellular K might be contained in or near the membrane, or might arise as the result of an inhomogeneous red cell population, while the larger comprising 95 per cent of the cellular K was contained within the cellular envelope. The fluxes were chosen so that the influx equalled the efflux across each interface, that is $k_n = k_{-n}$ for all values of n. It was, however, pointed out that an asymmetrical system in which this relation did not obtain would be adaptable to the unidirectional transport of a single ion. Further experience with the computer has indicated that an asymmetrical three compartment system, is, indeed, more compatible with the experimental data, and such a system has been adopted as the model system for the present experiments.

The defining equations for the present system are:

$$d(a\bar{p})/dt = -k_1\bar{p} - k_{-3}\bar{p} + k_{-1}\bar{q} + k_3\bar{r} \quad (1\,a)$$

$$d(b\bar{q})/dt = k_1\bar{p} - k_{-1}\bar{q} - k_2\bar{q} + k_{-2}\bar{r} \quad (1\,b)$$

$$d(c\bar{r})/dt = k_{-3}\bar{p} + k_2\bar{q} - k_3\bar{r} - k_{-2}\bar{r} \quad (1\,c)$$

in which $\bar{p}$, $\bar{q}$, and $\bar{r}$ represent the specific activities (relative counts per minute/millimols K) in compartments P, Q, and R respectively. a, b, and c represent the K content (millimols/liter blood)[1] in compartments P, Q, and R respectively. The k's represent the fluxes (millimols/liter blood hour) between the various compartments as indicated in Fig. 1. The assumptions inherent in compartmental analysis of tracer experiments have previously been discussed in detail (8) and are considered to apply to the present treatment.

In the steady state, a, b, and c are constants and the following relation exists among the k's:

$$\begin{aligned} k_1 - k_{-1} &= k_2 - k_{-3} \\ k_2 - k_{-2} &= k_3 - k_{-3} \end{aligned} \quad (2)$$

If we make the assumption that escape from the deep intracellular compartment, R, to the extracellular one, P, is a passive process, a further relationship can be adduced between k_3 and k_{-3}. This assumption is in agreement with the suggestion made by Tosteson (9) that there is a passive route for K escape from the red cell. Ussing (10) has shown that the ratio between influx and efflux through a passive canal is given by an equation of the following type:

$$\frac{k_3}{k_{-3}} = \frac{[\mathrm{K}]_r}{[\mathrm{K}]_p}\, e^{(zF/RT)(\psi_r - \psi_p)} \quad (3)$$

in which $[\mathrm{K}]_r$ and $[\mathrm{K}]_p$ represent the K concentration in millimols/liter water in compartments R and P respectively. z, the charge on the K ion, is equal to 1; F, is the Faraday; R, the gas constant; and T, the absolute temperature. $(\psi_r - \psi_p)$ is the potential difference across the cellular membrane which has been calculated to be -9.9 mv. (5). Equation 3 has been derived from the one given by Ussing by making the assumption that the activity coefficient inside the cell equals that outside, and by substituting our symbols for his. Using values of 94.1 mM K/liter cells and 4.65 mM K/liter plasma as previously given (5), a cell water content of 72.2 per cent, and plasma water content of 93.2 per cent as given by Hald *et al.* (11), the ratio $k_3/k_{-3} = 18.0$.

It has proved possible to devise a model system subject to the restrictions above whose performance on the analogue computer fits the experimental data for influx and efflux at least as well as the system previously given. Sub-

[1] In the present study, the term *blood* will be used in a generic sense for a suspension of cells in medium, although the medium may consist of plasma, dilute solutions of plasma, or buffer.

ject to the restraint that the k_3 canal be passive, it utilizes four independent adjustable constants, namely the $b/(b + c)$ ratio, k_1, k_2, and k_3. This increase of one constant over the number previously required for the symmetrical system provides increased flexibility, since it makes possible the uphill transport of K into the cell.

The following procedure was used in fitting the experimental data with the analogue computer:

The experimentally determined value of a was used.

Since b and c are not experimentally separable, the individual values were taken as:

$$b = 0.04\ (b + c)$$

$$c = 0.96\ (b + c)$$

All the k's except k_1 and k_{-1} were set in accordance with the values given in Table I, which represent those giving the best fit on the computer. The process of obtaining

TABLE I

Constants for Red Cell Model System

Constant	Flux
	mM/liter blood hr.
k_2	1.50
k_{-2}	1.00
k_3	0.54
k_{-3}	0.03

the best computer fit is necessarily a subjective one. It involves setting all the constants by trial and error until that combination is found which gives the best fit for experiments of influx and efflux, under all experimental conditions.

Each experiment was fitted individually, by adjustment of k_1 and k_{-1} subject to the constraint in the steady state:

$$k_1 - k_{-1} = 0.50 \text{ (mM/liter blood hour)}$$

The numerical value of this constraint arises from the value chosen for k_{-3}, which is the uphill passive flux, and the requirement that $k_3/k_{-3} = 18.0$. k_{-3} cannot be large, since if it were the passive downhill leak would overcome the ability of the cell to pump K uphill. A value of 0.03 mM/liter blood hour for k_{-3} appeared to give the best fit to the data.

In practice all the values on the computer except k_1 and k_{-1} were set initially. The experimental curves were printed on a 3.25 × 4 inch lantern slide, which was then clamped in front of the computer oscilloscope screen. After adjustment of the oscilloscope so that the axes coincided, the x and y amplification factors were adjusted to provide the correct time scale and a relative specific activity of 0.95 to 1.0 at $t = 0$. Then k_1 and k_{-1} were adjusted by eye, subject to the constraint above, to produce the best fit to the data over the rest of the experimental period.

Fig. 2 shows the fluxes and compartment sizes used in the fit for a typical case. It will be noted that the difference between k_1 and k_{-1} is greater than 0.50 mM/liter blood hour. This is a consequence of the slight gain in cellular K usually observed under normal conditions (6). The details of the calculation will be discussed below at the close of the section on non-steady state kinetics.

An example of the fit of the present asymmetric system to a wide variety of experimental conditions can be given by its application to the efflux of K^{42} from cells previously incubated for 2 hours with a large amount of K^{42} and then resuspended in fresh non-radioactive buffer solutions. In one case the cells were washed, and in the other they were not. The full experimental de-

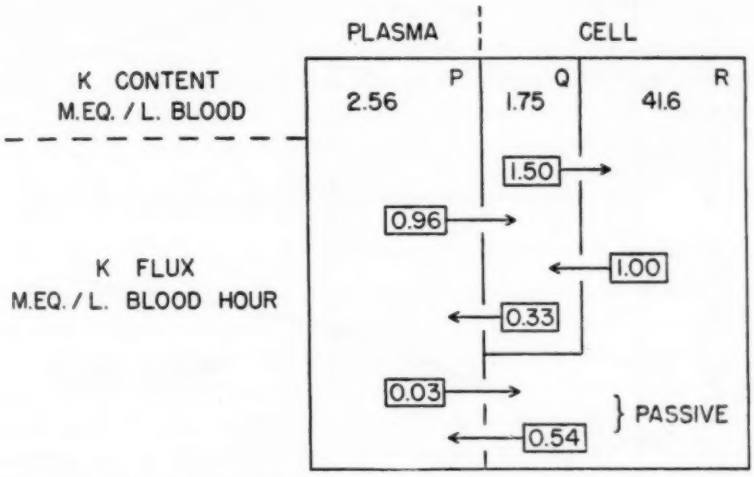

FIG. 2. Fluxes and compartment sizes for human blood under typical normal conditions.

tails, together with figures showing the computer fit to this data on the basis of a symmetrical three compartment system have been given by Solomon and Gold (7, Figs. 5 and 6). The fit of the present asymmetric model system to this old data is shown in Fig. 3. This fit is somewhat better than that previously obtained.

The approximately steady state conditions used to obtain the results given in Figs. 2 and 3 no longer obtain when one of the cardiac glycosides is used since the K concentration in the plasma rises rapidly under these conditions. Typically, in the course of one of our 4 or 5 hour experiments, the K concentration in the plasma doubles, rising in an essentially linear fashion, so that a, the K content in compartment P, may be expressed as follows:—

$$a = a_0(1 + \gamma t) \tag{4}$$

in which a_0 represents the initial value of a; γ, the fractional rate (in hours) at which a increases; and t, time. The concomitant loss from the cells of 4 to 5 mM K/liter cells over the course of the experiment is more difficult to treat theoretically. If the loss comes from the large intracellular compartment R, it would amount to only about 5 per cent of the K content, and hence can be

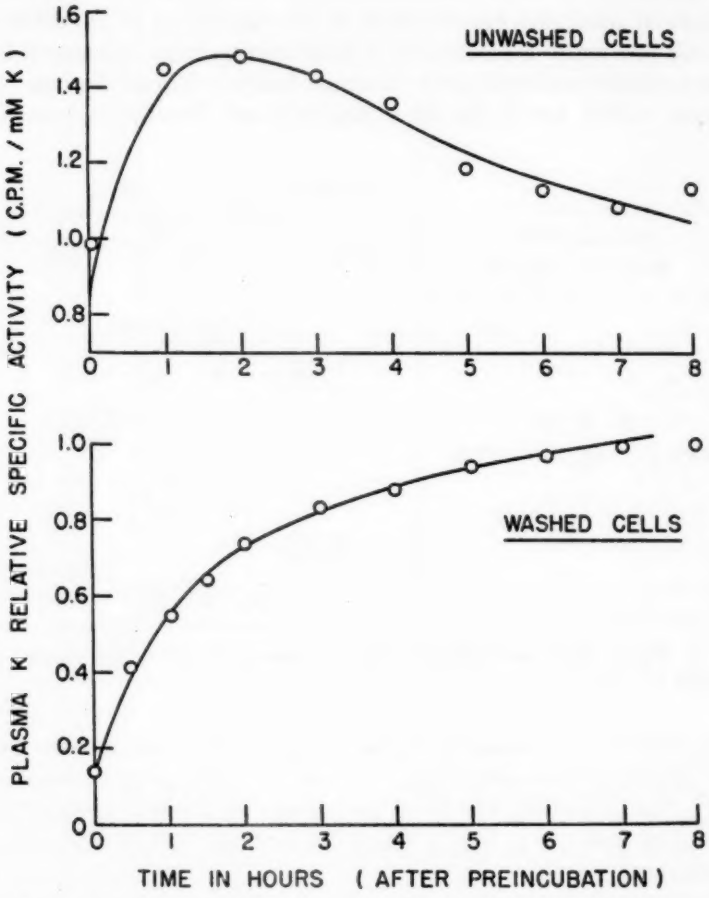

FIG. 3. Fit of analogue computer curves to experimental data for efflux of K^e from normal human red cells following 2 hour preincubation. The circles represent experimental data, and the curves are drawn from photographs of the oscilloscope screen of the computer. The upper curve was normalized at the 2 hour point. The lower curve was translated by 0.6 hour, so that the relative specific activity coincided with that observed at 0 time. This corresponds to a correction for the radioactivity lost from the Q compartment during the washing procedure. The translated curve was then normalized to a relative specific activity of 0.99 at 7 hours. It can be seen that the fit obtained in both cases is satisfactory.

neglected. Thus c can be treated as a constant. However, if the loss comes from the small compartment, Q, it would represent a large percentage change, so that b would have to be treated as a variable. In view of our inability to measure the K content of these two compartments separately, the question cannot now be resolved. Consequently, no compensation has been made for the loss of K from the cellular compartments and b has also been treated as a constant. If it turns out, as is not unlikely, that the loss does come from Q, values for the flux from this compartment under the influence of cardiac glycosides will have to be materially revised.

When a changes with time according to Equation 4, Equation 1 a becomes:

$$a_0(1 + \gamma t)(d\bar{p}/dt) = -k_1\bar{p} - k_{-3}\bar{p} + k_{-1}\bar{q} + k_3\bar{r} - a_0\gamma\bar{p} \quad (5)$$

Since b and c are treated as constants, Equations 1 b and 1 c do not have to be modified. With the addition of a divider to the normal array of computing elements, it becomes possible to use the analogue computer to solve the modified equations. The procedure already given in detail need be revised only by setting the value of γ into the computer in two places and altering the constraint on $k_1 - k_{-1}$ as follows:—

$$k_1 - k_{-1} = k_3 - k_{-3} - (da/dt) = 0.50 \text{ (mM/liter blood hour)} - (da/dt) \quad (6)$$

It has previously been shown (9) that under normal conditions of incubation the cells show a slight rise in K concentration. This is equivalent to a negative value for γ which has been set into the computer when it appeared to be significant. When ouabain is used at a concentration of $10^{-4.5}$ M/liter blood, the plasma gains K at a rate of 0.5 mM/liter blood hour and γ is positive. The computer fit to the data under these conditions, as shown in Fig. 4, is seen to be relatively good. The difference between the theoretical curve and the experimental data at zero time is characteristic.

Experimental Results

Effect of Cardiac Glycosides on K Influx.—

Six experiments were carried out to study the effect of ouabain on K influx at concentrations varying from 10^{-4} to 10^{-10} M/liter blood. The results of these experiments were interpreted on the analogue computer and are presented in Table II. Fig. 5 shows the experimental data for the fall in plasma relative specific activity in a typical ouabain experiment. Eight experiments were carried out on the effects of other cardiac glycosides on K influx. The results of these experiments, as interpreted by the analogue computer, are given in Table III.

Effect of Cardiac Glycosides on Glucose Consumption.—

Glucose consumption has been measured in almost all the experiments. The mean glucose consumption in thirteen control determinations is 1.56 ±

0.39 mM/liter cell hour. In measurements of glucose consumption with varying concentrations of glycosides, the mean glucose consumption in thirty-nine determinations is found to equal 1.53 ± 0.24 mM/liter cell hour, a figure which does not differ from the consumption of the control cells. This conclusion is in agreement with the initial finding of Schatzmann (1), who measured lactate production by cells and found no measurable effect of glycoside on this process. Glynn (3) has compared the effects of glucose deprivation and digoxin poisoning on both Na and K fluxes. From his studies, he has concluded that

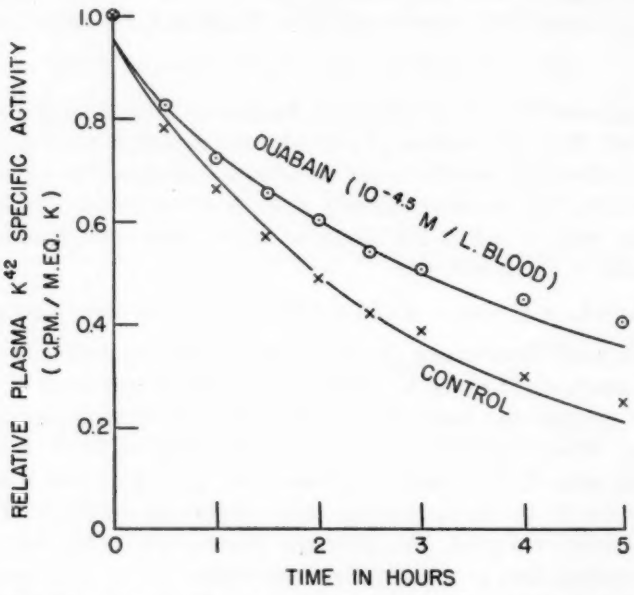

FIG. 4. The effect of ouabain ($10^{-4.5}$ M/liter blood) on K influx in human red cells. The points are experimental data, and the curves are obtained from photographs of the oscilloscope screen of the analogue computer.

digoxin does not uncouple the energy supply, but acts instead directly on the carrier mechanism.

DISCUSSION

Kinetic Hypothesis of Glycoside Action.—

Before proceeding to an interpretation of the results that have been obtained, it is necessary to present a kinetic hypothesis by which the data may be examined. Since the cardiac glycosides affect K flux without producing any apparent alteration in glucose metabolism or energy coupling, the kinetic scheme has been set up on the basis of competition of glycoside and K for a

TABLE II

Effect of Ouabain on K Influx into Human Red Cells

Experiment	Ouabain concentration	Initial plasma K concentration	Flux		Net rate of K loss from cells	Relative influx k_1 (drug) / k_1 (control)
			k_1	k_{-1}		
	$-\log_{10}$ M/liter blood	*mM/liter plasma*	*mM/liter blood hr.*			
M-3	∞ *	4.75	0.81	0.30	−0.04	
	5	5.00	0.12	0.24	0.54	0.15
	6	4.94	0.21	0.33	0.62	0.26
S-10	∞	4.78	1.02	0.42	−0.07	
	7	4.86	0.51	0.39	0.39	0.50
	8	4.82	0.90	0.54	0.11	0.88
	9	4.91	0.93	0.33	−0.07	0.91
S-11	∞	5.00	0.69	0.09	−0.05	
	4	5.04	0.12	0.03	0.43	0.17
	5	5.14	0.15	0.03	0.39	0.22
	6	5.13	0.18	0.06	0.39	0.26
S-13	∞	4.55	0.99	0.39	−0.14	
	4.5	4.74	0.18	0.12	0.50	0.18
	5.5	4.80	0.27	0.21	0.50	0.27
	6.5	4.62	0.32	0.27	0.51	0.32
S-14	∞	5.18	0.93	0.39	−0.06	
	8	5.14	0.90	0.48	0.08	0.97
	9	5.04	0.93	0.39	−0.05	1.00
	10	5.04	0.93	0.39	−0.08	1.00
S-15	∞	4.64	1.02	0.45	−0.06	
	4	4.98	0.27	0.24	0.51	0.26
	5	4.85	0.27	0.21	0.46	0.26
	6	4.88	0.24	0.24	0.46	0.26

* This is equivalent to zero concentration of ouabain.

site on the cell surface (or for a K carrier, limited in amount). A simple kinetic scheme may be constructed on the basis of the following two reactions:

$$[K]_p + [S] \rightleftarrows [KS] \tag{7}$$

$$[G] + n[S] \rightleftarrows [GS_n] \tag{8}$$

in which $[K]_p$ refers to the plasma K concentration, expressed in units of M/liter blood, $[G]$ refers to plasma glycoside concentration in the same units, and $[S]$ refers to the first hypothetical reactant that is of importance in the entrance of K into the cell, in the same units. The initial S has been chosen to stand for site, so that KS represents the K, site complex which must be

formed before K is handed over for onward transport. Alternatively KS may represent the union of K with a carrier which is present in limited amount on the surface or in the membrane phase. The exact identity of KS, though a matter of the utmost moment in unraveling the intimate details of K kinetics, is unimportant for the solution of the kinetic equations; it is only necessary that it exist and that S be limited in amount. These reservations are meant to apply to the further discussion of S, though S will, for convenience, be referred to hereafter as a site.

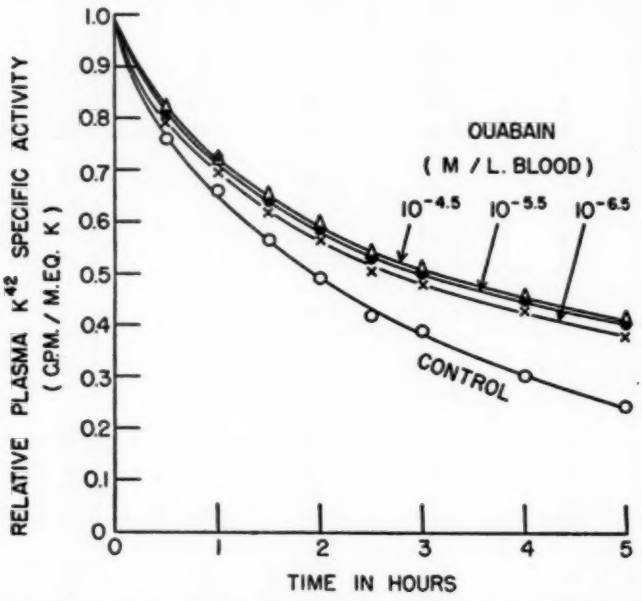

FIG. 5. Typical experiment showing the effect of ouabain on K influx in human red cells. The points are experimental data, and the curves have been drawn by eye to connect the points.

In the earlier stages of the present study, it appeared essential for the glycoside to react with more than one molecule of S in order to fit the experimental data with an expression derived from Equations 7 and 8. As the experimental data accrued, however, this requirement no longer held, and it was possible to obtain a satisfactory fit of theory to experiment on the basis of a 1:1 relationship between glycoside and S. Nonetheless, the kinetics will be developed on the basis of Equation 8, in which n can have values different from 1. Under these conditions, the equations of conservation are:

$$[S]_t = [S] + [KS] + n[GS_n] \tag{9}$$

$$[G]_t = [G] + [GS_n] \tag{10}$$

TABLE III

Effect of Cardiac Glycosides on K Influx into Human Red Cells

Experiment	Drug	Drug concentration	Initial plasma K concentration	Flux		Net rate of K loss from cells	Relative influx k_1 (drug) / k_1 (control)
				k_1	k_{-1}		
		−log₁₀ M/liter blood	*mM/liter plasma*	*mM/liter blood hr.*			
S-1	Lanatoside C	∞	5.72	1.26	0.66	−0.15	
		5	6.10	0.30	0.24	0.54	0.24
		6	5.68	0.48	0.42	0.46	0.38
		7	5.58	0.90	0.54	0.11	0.71
S-2	Digoxin	∞	4.76	1.02	0.51	−0.04	
		5	4.91	0.15	0.12	0.50	0.15
		6	4.79	0.27	0.21	0.47	0.27
		7	4.88	0.48	0.24	0.26	0.47
S-3	Digoxigenin	∞	4.53	1.26	0.69	−0.08	
		5	4.67	0.36	0.27	0.48	0.29
		6	4.62	0.45	0.21	0.28	0.36
		7	4.50	0.93	0.45	0.04	0.74
S-4	Strophanthidin	∞	4.89	0.87	0.33	−0.04	
		5	5.03	0.18	0.18	0.60	0.21
		6	4.98	0.39	0.42	0.53	0.45
		7	4.88	0.69	0.33	0.18	0.79
S-5	Strophanthidin acetate	∞	4.60	0.57	0.00	−0.08	
		5	4.84	0.12	0.00	0.42	0.21
		6	4.84	0.21	0.00	0.32	0.37
		7	4.76	0.17	0.30	0.21	0.90
S-6	Digitoxigenin	∞	4.72	0.90	0.27	−0.14	
		5	4.91	0.21	0.09	0.45	0.23
		6	4.79	0.51	0.27	0.24	0.57
		7	4.74	0.81	0.30	−0.05	0.90
S-7	Lanatoside B	∞	4.87	0.81	0.21	−0.07	
		5	4.98	0.36	0.18	0.36	0.44
		6	4.84	0.66	0.27	0.12	0.82
		7	4.86	0.96	0.45	−0.01	1.18
S-8	Scillaren A	∞	5.86	0.81	0.21	−0.10	
		5	6.18	0.24	0.15	0.38	0.30
		6	5.96	0.27	0.12	0.35	0.33
		7	5.81	0.51	0.21	0.23	0.63

in which the subscript t refers to total amount. The equilibrium constants, K_k and K_G, are given by:

$$\frac{[KS]}{[K]_p[S]} = K_k \tag{11}$$

$$\frac{[GS_n]}{[G][S]^n} = K_G \tag{12}$$

For Equation 11 to be valid, it is necessary to assume that the reaction for onward transfer of K from KS be slow compared with the back reaction, the dissociation of KS into K_p and S. This assumption is consistent with the relative independence of K transport and plasma K concentration and the equations that have previously been derived to describe the relationship between these quantities (5, 6). Let us define the constants α and β as follows:—

$$\alpha = [KS]/[KS]_0 = k_1/k_{1.0} \tag{13}$$

$$\beta = (1 + K_k[K]_p) \tag{14}$$

in which the subscript 0 refers to the absence of glycosides. $k_{1.0}$ is the flux of K from P to Q in the control measurements, and k_1 the P to Q flux in the presence of glycosides. Next let us relate $[KS]_0$ to $[S]_t$ when $[S] = [S]_t - [KS]_0$, that is, in the absence of glycoside.

$$\frac{[KS]_0}{[K]_p([S]_t - [KS]_0)} = K_k \tag{15}$$

$$[KS]_0 = (1/\beta)K_k[K]_p[S]_t \tag{16}$$

and in the presence of glycoside:

$$[KS] = \alpha[KS]_0 = (\alpha/\beta)K_k[K]_p[S]_t \tag{17}$$

substituting 17 in 11 we obtain:

$$[S] = (\alpha/\beta)[S]_t \tag{18}$$

substituting 17 and 18 in 9 and rearranging, we obtain:

$$[GS_n] = (1/n)(1 - \alpha)[S]_t \tag{19}$$

substituting 19 in 10 and rearranging, we obtain:

$$[G] = [G]_t - (1/n)(1 - \alpha)[S]_t \tag{20}$$

and substituting 18, 19, and 20 in 12 and rearranging, we obtain:

$$[G]_t = \frac{\beta^n}{K_G[S]_t^{n-1}n}\frac{(1 - \alpha)}{\alpha^n} + \frac{(1 - \alpha)[S]_t}{n} \tag{21}$$

letting $n = 1$, and eliminating β by the use of 14, this reduces to:

$$[G]_t = \frac{(1 + K_k[K]_p)(1 - \alpha)}{K_G\,\alpha} + (1 - \alpha)[S]_t \tag{22}$$

This treatment is completely analogous to that given by Goldstein (12) for inhibition of enzyme reactions. The first term on the right represents the free glycoside, and the second term represents the glycoside complexed with S.

Equation 22 has been derived on the assumption that ouabain completely inhibits K influx. However, an examination of the figures in column 7 of Table II reveals that in no case has complete inhibition been obtained, and that about 20 per cent of the influx is independent of the drug. Consequently in fitting the data to Equation 22, α has been normalized to α' according to the following equation:

$$\alpha' = 1.25(\alpha - 0.2) \tag{23}$$

The values of α are given as $k_1/k_{1,0}$ in the last column of Table II. Apparently individual bloods vary considerably in the exact fraction of influx that may be inhibited by the drug; this is reflected by the negative values of α' obtained in some experiments. Substitution of α' in Equation 22 leads to:

$$[G]_t = \frac{(1 + K_k[K]_p)(1 - \alpha')}{K_G \alpha'} + (1 - \alpha')[S]_t \tag{24}$$

This equation has been used to derive the theoretical curve shown in Fig. 6 which relates the values of α' to ouabain concentration. It can be seen that the theory fits the data in a satisfactory way. The conclusion that the system is saturated with ouabain at concentrations of 10^{-4} M/liter blood may be confirmed from an examination of Fig. 5, which shows the effect of ouabain in concentrations of $10^{-4.5}$, $10^{-5.5}$, and $10^{-6.5}$ M/liter blood. The results of Kahn and Acheson (4) on ouabain inhibition of K uptake by cold stored red cells show a similar saturation effect at somewhat lower concentrations.

The fit that has been obtained in Fig. 6 makes use of only the first term (free glycoside) on the right hand side of Equation 24, since the inclusion of the second term (bound glycoside) makes the fit worse. This means that in spite of the very great affinity of the glycosides for the cell surface, the bound glycoside represents only a negligible fraction of the total glycoside present.

When $\alpha' = 0.5$, and $(1 - \alpha')/\alpha' = 1$, $\log_{10}[G]_t$ is equal to -7.25. In the absence of a contribution from the second term in Equation 24, $[G]_t$ may be set equal to $(1 + K_k[K]_p)/K_G$. K_k may be evaluated from the influx data presented by Streeten and Solomon (6) who have shown that the influx depends upon plasma K concentration according to the following equation:

$$\text{K influx (mM/liter cell hour)} = [K]_{pi}/(0.697 + 0.329\ [K]_{pi}) \tag{25}$$

in which $[K]_{pi}$ refers to the initial plasma K concentration in millimols/liter plasma. Equation 25 is equivalent to:

$$\text{K influx} = \frac{1.43[K]_p v_g}{v_p(1 + 472[K]_p/v_p)} \tag{26}$$

in which v_p and v_q are the relative fractional volumes of plasma and cells respectively, K influx is expressed in terms of mols/liter blood hour, and $[K]_p$ is expressed as mols/liter blood. In the absence of glycoside, as was the case in the experiments of Streeten and Solomon, K influx must also be proportional to $[KS]_0$, and hence from 16 and 14:

$$\text{K influx} = (\epsilon K_k[K]_p[S]_t)/(1 + K_k[K]_p) \tag{27}$$

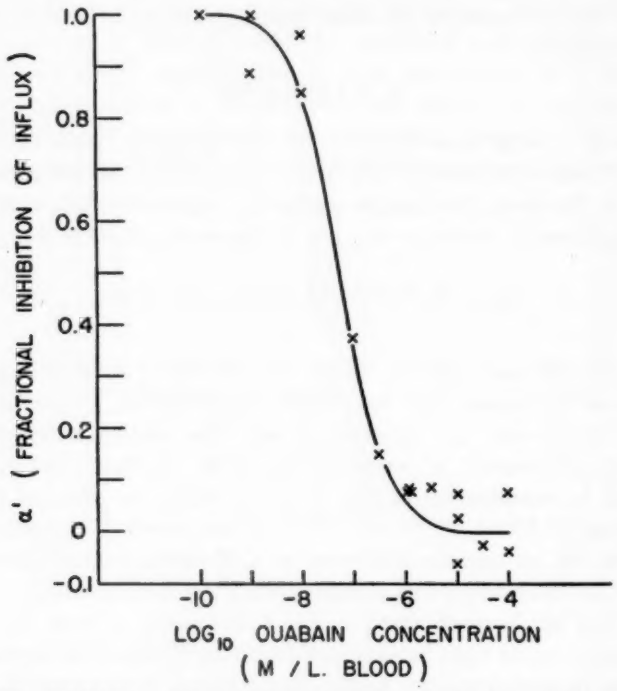

FIG. 6. The inhibition of K flux into human red cells by ouabain. The points represent experimental data, and the curve has been derived from Equation 24 as described in the text.

in which ϵ is the proportionality constant which represents the fraction of $[KS]_0$ that hands over its K in unit time. By inverting Equations 26 and 27, and comparing terms in like powers of $[K]_p$, it can be shown that $K_k = (472/v_p)$ liters blood/M. Since the average value of v_p for all the experiments shown in Table III is 0.551, K_k is equal to 860 liters blood/M. Using this value and the observed mean plasma K concentration in the same experiments, 2.71×10^{-3} M/liter blood, the association constant, K_G, may be evaluated as 5.91×10^7 liters blood/M. Although this value may not be compared directly with the results of Kahn and Acheson (4) who measured the ouabain inhibition of K

uptake by human red cells that had previously been cold stored, it is possible to compare the present figure for the ouabain concentration required to reduce α' to 0.5 with their "pI_{50}". The agreement between these values is very good, since they obtain 50 per cent inhibition with a concentration of 6.8×10^{-8} M/liter blood, which is to be compared with a concentration of 5.6×10^{-8} M/liter blood required to reduce α' to 0.5 in the present study.

Since Equation 24 seems to offer satisfactory agreement between experiment and theory with respect to the action of increasing concentrations of glycoside on K influx, it should be possible to extend the treatment to a prediction of net K loss under the influence of glycosides. In the normal cell:

$$\text{Net K influx}_{(0)} = \text{K influx}_{(0)} - \text{K efflux}_{(0)} \tag{28}$$

in which the subscript (0) refers to fluxes in the absence of glycoside. A similar equation may be written for the fluxes under the influence of glycoside, in which case the subscript (g) is used. Let us next assume that the glycosides have no effect on K efflux, so that $\text{K efflux}_{(0)} = \text{K efflux}_{(g)}$. This assumption has not been tested in the present study, since efflux experiments in a three compartment system are particularly difficult of interpretation. However, Glynn (3) is of the opinion that the glycoside, digoxin, also exerts an influence on K efflux reducing it by about 25 per cent. If, for simplicity, this effect is neglected, we may write:

$$\text{Net K influx}_{(0)} - \text{net K influx}_{(g)} \cong \text{K influx}_{(0)} - \text{K influx}_{(g)} \tag{29}$$

Let us call the expression on the left hand side of the equality sign Δ net K loss. As already pointed out, K influx in the presence and absence of glycoside may be obtained from Equations 16 and 17, since it must be proportional to $[KS]$. Rewriting Equation 27 we obtain:

$$\text{K influx}_{(0)} = \epsilon[KS]_0 = \epsilon(1/\beta)K_k[K]_p[S]_t \tag{30}$$

and similarly:

$$\text{K influx}_{(g)} = \epsilon[KS] = \epsilon(\alpha/\beta)K_k[K]_p[S]_t \tag{31}$$

Now, substituting 30 and 31 in 29 we may write:

$$\Delta \text{ net K loss} \cong (1 - \alpha)(\epsilon/\beta)K_k[K]_p[S]_t \tag{32}$$

Since $[K]_p$ is approximately constant for the ouabain experiments, Δ net K loss should be proportional to $(1 - \alpha)$.

The relationship between $(1 - \alpha)/\alpha$ and $[G]_t$ is given by Equation 22, neglecting the second term. The coefficient relating these two terms, $(1 + K_k[K]_p)/K_G$, has already been evaluated from the curve shown in Fig. 6. Thus $[G]_t$ may be calculated for any value of $(1 - \alpha)$. The final step necessary to obtain the curve relating Δ net K loss to $[G]_t$, as shown in Fig. 7, was the determination of the proportionality constant relating Δ net K loss to $(1 - \alpha)$

in Equation 32. This constant was chosen in order to provide the best visual fit of the curve to the experimental data, which is obtained, with considerable uncertainty, when Δ net K loss equals 1.33 mM/liter cell hour at a ouabain concentration of 10^{-4} M/liter blood. A Δ net K loss of 1.33 mM/liter cell hour under conditions of maximal inhibition corresponds to 63 per cent inhibition, since the normal K influx at these plasma K concentrations is 2.1 mM/liter cell hour (6). This is appreciably less than the figure of 80 per cent inhibition

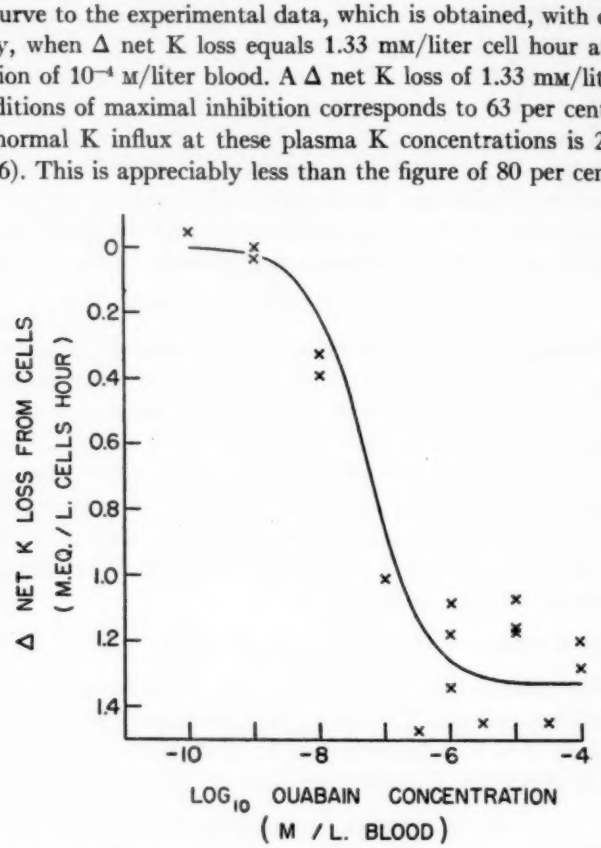

FIG. 7. Net K loss from human red cells caused by ouabain. The points represent experimental data, and the curve has been derived from Equation 32 as described in the text.

obtained from the kinetic data; the difference may possibly be ascribed to the effect of the glycoside on K efflux observed by Glynn (3). Nonetheless, it is heartening to observe that kinetic predictions based on computer data agree relatively well with directly measured cellular K losses.

Characteristics of Normal Cell Derived from Inhibition Studies.—

The concentration of binding sites on the cell surface, $[S]_t$, cannot be obtained directly from Equation 24 since the term involving $[S]_t$ can be neglected. However, an estimate of its maximum value may be obtained from the value of $[S]_t$ required to displace the curve in Fig. 6 by an amount large enough to obtain a demonstrably worse fit to the data. This procedure leads to an esti-

mate that $[S]_t \leqq 10^{-7}$ M/liter blood which on the basis of a red cell population of 5×10^6 cells/mm.^{3} blood is equivalent to 1.2×10^4 sites per cell. Accepting Ponder's figure of 167 μ^2 for red cell surface area (13), the population is 72 sites/μ^2, which is the approximate equivalent of 1 site/10^6 A^2, a rather sparse population indeed.

We can also use the maximum estimate of $[S]_t$ to give us a maximum value for $[KS]_0$ from Equation 16 of 0.69×10^{-7} M/liter blood. From Equations 26 and 27 it can be shown that:

$$\epsilon K_k[S]_t = 1.43\, v_q/v_p \tag{33}$$

Using the values of v_p, K_k and $[S]_t$ given above, we may calculate that $\epsilon \geqq 1.2 \times 10^4$, or that each KS site passes a K atom along somewhat more frequently than 10,000 times an hour.

TABLE IV

Effect of Increased Plasma K Concentration on K Influx in Ouabain-Treated Red Cells (10^{-6} M Ouabain/Liter Blood)

Plasma K concentration	Flux		Net rate of K loss from cells
	k_1	k_{-1}	
mM/liter plasma	*mM/liter blood hr.*		
4.02	0.21	0.21	0.49
7.20	0.36	0.27	0.41
10.3	0.48	0.39	0.43
13.6	0.42	0.30	0.35

Effect of Plasma K Concentration on Ouabain Inhibition.—

Equation 24 predicts that increases in plasma K concentration will decrease the effective glycoside inhibition. This prediction has been tested experimentally in a qualitative fashion, and the results are given in Table IV. It will be seen that an increase in plasma K concentration from 4.02 to 13.6 mM K/liter plasma results in doubling the K influx when the cell has been treated with ouabain at a concentration of 10^{-6} M/liter blood. Quantitative correlation of this increase with that predicted by Equation 24 is not possible, since we do not know either the exact relationship between α and α' for this experiment, nor do we have a theoretical expression which predicts the dependence of the drug-insensitive influx on plasma K concentration.

A similar competition between K and the action of cardiac glycosides has been discussed by Lown and Levine (14) who have summarized observations that certain effects of digitalis on the heart in man and experimental animals may be diminished by the administration of K. These observations suggest that the mechanism of action of these drugs on the heart may be similar in some respects to their action on red cells.

Glycoside Removal by Washing Cells.—

Two experiments were carried out to measure the ease of removal of the drug from the cell by washing. Since these experiments were of necessity carried out at a period when the K specific activity was lower than normal, it was necessary to increase the plasma K concentration to provide enough K^{42} to count. The blood was incubated for 1 hour with the drug, washed three times with buffer (Table I, reference 7), and then incubated with K^{42} in the usual fashion. The results given in Table V show that under these conditions ouabain is only removed from the cell surface with great difficulty, whereas digitoxigenin is removed with ease. This result is in agreement with the suggestion of Straub (15) that the presence of the sugar moiety of the molecule results in a tighter binding of the glycoside to the cell surface. The fact that

TABLE V

Effect of Washing on Removal of Drug from Red Cells

Experiment	Drug	Concentration	Initial plasma K concentration	Flux		Net rate of K loss from cells	Relative influx $\frac{k_1 \text{ (drug)}}{k_1 \text{ (control)}}$
				k_1	k_{-1}		
		M/liter blood	mM/liter plasma	mM/liter blood hr.			
W-1	Ouabain	0	13.9	1.50	0.51	−0.05	
		10^{-6}	12.0	0.96	0.92	0.07	0.64
W-2	Digitoxigenin	0	8.13	0.72	0.06	−0.17	
		10^{-6}	8.25	0.69	0.06	−0.11	1.04

the inhibition of the K influx by ouabain shown in Table V is much less than that expected from an examination of Fig. 6 may be fairly ascribed to the high K concentration in Experiment W-1.

As has been discussed, the association between glycoside and red cell can be described in terms of the association constant, K_G, which, for ouabain, has the value, 59.1×10^6 liters blood/M. It will be shown in the succeeding section that the value of K_G for digitoxigenin is more than ten times smaller than that for ouabain, namely, 4.11×10^6 liters blood/M. Thus it is to be expected that ouabain will be much more difficult to remove from the cell by washing than digitoxigenin.

Comparison of Cardiac Glycoside Effect on Red Cell K Transport with Mean Lethal Dose in Cats.—

In addition to ouabain, the effect of eight other glycosides on red cell K transport has been examined. For this purpose, the glycosides were used in uniform concentrations of 10^{-5}, 10^{-6}, and 10^{-7} M/liter blood. For each glyco-

side a dose response curve has been drawn, based on the values of k_1 obtained from the computer. The ratio α has been normalized to α' according to Equation 23. This involves the assumption that the fraction of the influx unaffected by ouabain is constant, which as we have seen is only approximately correct, and which will lead to errors that increase as α approaches 0. K_G is determined by the use of Equation 24 (neglecting the second term) from the value of $[G]_t$ when $\alpha' = 0.5$. The inhibition in one exceptional case, that of digoxin, was so marked that no observations were made with α' as large as 0.5, and K_G was determined from the value of $[G]_t$ for $\alpha' = 0.3$. The values of all nine association constants, K_G, are summarized in Table VI, together with the values of the dissociation constant, $(K_G)^{-1}$, and the mean lethal dose of these same glycosides for cats as determined by Chen (16), and converted to units

TABLE VI

Comparison of Cardiac Glycoside Mean Lethal Dose in Cats with Effect on Red Cell K Transport

Glycoside	K_G Association constant	K_G^{-1}	Mean lethal dose in cats
	liters blood/μ $\times 10^{-6}$	*m/liter blood* $\times 10^{6}$	μ*m/kg.*
Ouabain	59.1	1.69	0.159
Digoxin	55.3	1.81	0.296
Scillaren A	32.4	3.09	0.210
Lanatoside C	20.9	4.78	0.236
Digoxigenin	19.5	5.13	1.13
Strophanthidin acetate	13.5	7.41	0.417
Strophanthidin	10.1	9.90	0.804
Digitoxigenin	4.11	24.3	1.225
Lanatoside B	0.87	115	0.393

of micromols/kilo body weight. The relation between these two sets of values is shown graphically in Fig. 8. It will be noted from Table VI that only three glycosides are out of order in the stepwise progression from greatest to least effectiveness; digoxin, digoxigenin, and lanatoside B. From Table III, it can be noted that α for digoxin at a concentration of 10^{-5} M/liter blood is 0.15, and that consequently α' has a negative value. Since this drug is also so effective that the determination of K_G was made for $\alpha' = 0.3$ instead of 0.5, the discrepancy between Chen's figures and the present one for digoxin may be ascribed to errors inherent in the kinetic analysis.

On the other hand the differences in the cases of digoxigenin and lanatoside B are so large that they must be real. We might tentatively suggest that in the case of digoxigenin, a detoxification mechanism exists in the whole animal that is absent in the red cell, for the effectiveness of digoxin and lanatoside C (which both contain digoxigenin as aglycone) attests to the fact that the

aglycone, with the proper combination of sugar and acetate groups, can have a considerable effect on the cat. Since these latter two drugs are also effective in the red cell, the diminished sensitivity of the cat to digoxigenin suggests that in the whole animal a protective mechanism exists that is much less effective when the aglycone is coupled to its sugar. One function of the sugar moiety appears to be that of holding the aglycone firmly to the cell surface as has been previously pointed out in connection with our washing experiments, so a bound glycoside might well be much more resistant to detoxification than one floating freely in solution. The existence of a detoxification

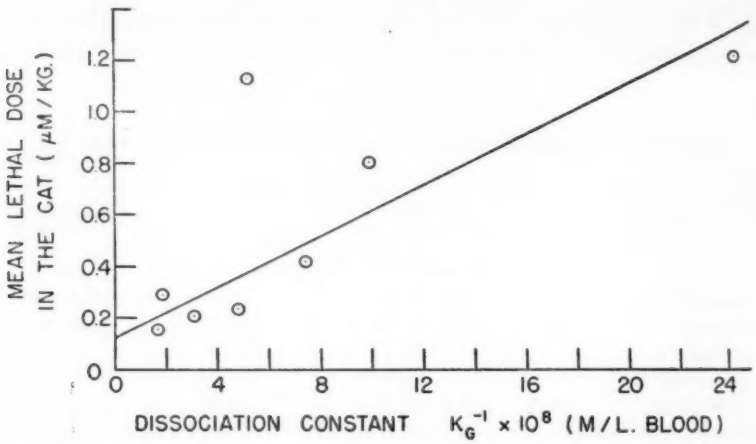

FIG. 8. The relationship between the effect of eight cardiac glycosides on the cat, as evidenced by the mean lethal dose, and the dissociation constant between the glycosides and sites on the human red cell. The points are derived from experimental data, and the curve has been drawn by eye. The point for lanatoside B is not included in the graph, since it would be a great distance off the scale.

mechanism may be inferred from the experiments of Okita *et al.* (17) who have shown a continued excretion of both digitoxin and its metabolic products following the administration of C^{14} digitoxin to man.

In the case of lanatoside B, the considerable effectiveness of the glycoside is apparent from the experiments in the cat. However, it is by far the least active glycoside tested on the red cell. The difference between the two is far outside of experimental error and is at present unexplained.

The most striking conclusion that may be drawn from this comparison has to do with the similarities rather than the differences between the red cell in man, and the heart in the cat. It appears clear that the events leading to the death of the cat are accompanied by a serious derangement in the cation control mechanism, although whether this is a primary cause or a secondary

result is not yet apparent. Secondly, since with two exceptions glycoside effectiveness in cats is proportional to the interference with red cell cation transport, it appears probable that the cation transport mechanisms operate in a similar fashion. Thus we might hope to find that the essential details of the cation transport mechanism in red cells would be not unlike those in heart muscle, and that the molecule or molecules most directly responsible would be similar in character.

TABLE VII

Effect of Corticosteroids on K Influx into Human Red Cells

Experiment	Drug	Dose	Corrected K influx
		M/liter blood	*mM/liter cell hour*
M-8	Fluorohydrocortisone	0	1.84
		10^{-7}	1.84
		10^{-6}	1.94
		10^{-5}	1.84
M-9	Electrocortin	0	1.53
		10^{-7}	1.63
		10^{-6}	1.55
		2.5×10^{-6}	1.66
S-18	17-hydroxycorticosterone	0	2.17
		7.2×10^{-6}	2.15
		3.6×10^{-5}	1.99
		7.2×10^{-5}	1.96

Effect of Corticosteroids on K Influx.—

The molecular specificity of the K transport system may well be illustrated by comparison of the results already given for the glycosides with those obtained with corticosteroids. Streeten and Solomon (6) have already shown that the effect of 17-hydroxycorticosterone on K influx is slight. In the present study, experiments in the same dose range have been carried out with fluorohydrocortisone and electrocortin,[2] kindly provided by The Squibb Institute for Medical Research and the Ciba Pharmaceutical Company. The results obtained with these latter two drugs in the dose range of 10^{-7} to 10^{-5} M/liter were calculated on the basis of a two compartment system as used for 17-hydroxycorticosterone. They are given in Table VII, together with the results of one of the earlier experiments with 17-hydroxycorticosterone. It will be seen that no effects are apparent with either fluorohydrocortisone or electrocortin, and that the effect is real but small with 17-hydroxycorticosterone.

[2] Electrocortin is now commonly known as aldosterone.

Since these drugs are similar in structure to the glycosides, it appears that the cation transport mechanism is very specific with respect to the molecular structure of its inhibitors. It seems probable that both the characteristic lactone ring of the glycosides and the exact nature of the other functional groups attached to the steroid nucleus play a part in determining the effectiveness of the inhibition. Glynn (3) and Kahn and Acheson (4) have come to a similar conclusion from their studies of lactones and other compounds with related structures.

Conclusions with Respect to the Mechanism of Cation Transport in Red Cells.–

Certain conclusions can be drawn from the present results which help to illuminate the problem of red cell cation transport. First the present model system supposes an active transport of K into the cells, and a passive route for K escape. This is in agreement with the hypothesis advanced by Tosteson (9), and is a simplification of the earlier hypothesis put forward by Solomon (5), which had included an additional active process for the efflux of K from the cell. The need for active efflux was based on the observed apparent activation energy for K efflux of 12,300 cals./mol, a value compatible with the activation energy of chemical reactions, and much greater than that found for free diffusion in water solution. However, as pointed out by Maizels (18) and Tosteson (9), high activation energies are found in certain chemical systems involving the penetration of lipide barriers by diffusion. As a consequence, the requirement that the outward transport be active has been dropped.

The present system also has more modest energy requirements than those of the earlier hypothesis. The energy required may be calculated in a typical case from the passive K efflux, which is 0.54 mM K/liter blood hour, as shown in Fig. 2. It is assumed that the cell is in the steady state, and is neither gaining nor losing K, and that all reactions are reversible, except for the passive K efflux, which is assumed to be irreversible. It has been shown (5) that the net free energy required for the inward transport of 1 mol of K into the red cells at 37°C. is 1644 calories. Since it is only necessary to replace the energy lost by the irreversible process, 0.89 calory/liter blood hour (or 1.96 calories/liter cell hour) is required to move 0.54 mM of K from plasma into the cell. The free energy available from glucose consumption in the red cell has been calculated by Bernstein (19) to be 28 calories/liter cell hour at 37°C. Thus on the basis of the assumptions given above only 7 per cent of the energy available from the glucose consumption is required for K transport.[3]

The observation that 20 per cent of the K influx is unaffected by glycosides, of whatever concentration, implies that there are two separate processes of

[3] The figure previously given by Solomon (5) for the energy available from glucose consumption was in error because of neglect of white cell glucose consumption, and the use of an erroneous figure for free energy available from glycolysis.

K influx, a concept that has not yet been introduced into the present working hypothesis. However, the hypothesis already embraces a great many assumptions that have not yet been put to experimental test. These assumptions should be firmly established before incorporating a process that only involves 20 per cent of the reactants into a definitive reaction scheme. Nonetheless, any final set of reactions which is to account for K entrance into the red cell must include an explanation for the fact that 20 per cent of the K influx is independent of the action of glycosides.

Certain conclusions emerge from our investigation of the action of the glycosides on red cell K transport. First, it is possible to account for the action of the glycosides on K transport on the basis of competition of glycoside and K for a substrate which is limited in amount, possibly a site on the cell surface. Second, the kinetics of this competition permit an estimate of the maximum number of sites, or substrate molecules, per cell and of the number of times each site passes its K onward for transmission into the cell. The affinity of the glycoside for the site has been expressed in terms of an association constant, and such constants have been determined for nine glycosides and aglycones. From the relationship of these constants to the mean lethal glycoside dose for the cat it appears that the action of the drug on red cells is in many respects similar to its action on the heart of the living animal.

We should like to express our thanks to Dr. John A. Johnson for introducing us to the glycoside problem, to Miss Betsy Manning for her important contributions in the early phases of the study, and to the Misses Jean McCormick, Daphne Adams, and Dorothy Driscoll for their help in counting and preparing the isotopes.

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METABOLISM OF EXCISED RAT SKIN IN HYPERTONIC MEDIA*

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(Received for publication, July 24, 1956)

There is considerable evidence indicating that freezing damages mammalian tissues by removal of water as ice; tissue damage is then attributable to the concentrated electrolyte solutions produced by this removal of water (1, 2). This hypothesis should be useful in understanding the tissue damage which occurs in experimental frost-bite. The rates of both oxygen consumption and anaerobic glycolysis of skin removed from frost-bitten feet of animals immediately after thawing are lower than in uninjured skin (3). QO_2 and $Q_G^{N_2}$ are also greatly reduced below control values by freezing skin *in vitro* (3). Explanation of these results on the basis of damage produced by high salt concentration requires information concerning the effect of hypertonic solutions on QO_2 and $Q_G^{N_2}$ of normal skin. Such information is given in this paper.

Methods

Skin from the dorsal surface of the hind feet of Wistar albino rats was used without slicing as previously described (4). Skin from this area is thin enough to permit adequate oxygen diffusion at 37.5°C. After removal the skin was blotted rapidly on filter paper, cut lengthwise into two, or occasionally three, pieces, weighed on a microtorsion balance, and put into respirometer vessels containing the desired medium. Oxygen consumption was determined by the direct method of Warburg using 100 per cent oxygen as the gas phase. Anaerobic glycolysis was measured as CO_2 liberated from bicarbonate, using 5 per cent CO_2–95 per cent N_2 (passed over hot copper) as the gas phase. The temperature was 37.5°C. in all experiments reported here. Unless otherwise specified, the rate of oxygen consumption or glycolysis was calculated from readings taken during the period from 60 to 90 minutes after placing the tissue in the various media. Where rates of oxygen consumption (QO_2) or anaerobic glycolysis ($Q_G^{N_2}$) are expressed in absolute terms they refer to microliters of gas consumed or produced, per milligram initial wet weight per hour.

The composition of the media used in the control experiments, and to supply balanced salt concentration in the modified media, is given in Table I. These media were based on Krebs's (5) medium III, but contain glucose as the only substrate.

* This investigation was supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-459.

Hypertonicity was obtained with NaCl, KCl, or sucrose. Concentrated hypertonic media were prepared in which the 0.154 M NaCl in medium A or medium F was replaced by an equal volume of 3.08 M NaCl or 3.08 M KCl. Sucrose was dissolved in medium A to give a final concentration of 3.74 M. The concentrated media and the isotonic media were then mixed in suitable proportions to give the various hypertonic media used for measurement of tissue metabolism.

TABLE I

Composition of Isotonic Media

	Concentration	Medium A	Medium F
	M	ml.	ml.
NaCl	0.154	110	95
KCl	0.154	4	4
$CaCl_2$	0.11	3	3
KH_2PO_4	0.154	1	1
$MgSO_4$	0.154	1	1
$NaHCO_3$	0.154	3	21
Phosphate buffer*	0.1	3	—
Glucose	0.3	5	5

* 100 ml. 0.1 M Na_2HPO_4 + 25 ml. 0.1 M NaH_2PO_4.

RESULTS

Effects of hypertonic media on the rate of oxygen consumption of rat skin are shown in Fig. 1. It has been shown previously that QO_2 of rat foot skin is dependent upon animal weight (4). For this reason the rats used for the experiments in Fig. 1 were within a restricted weight range (129 to 193 gm., mean = 157 gm.), but even this range of weight probably contributes to the variability of the data. With concentrations up to about 1.3 osmols per liter the inhibition was fully developed within 30 minutes after placing the tissue in the hypertonic medium; during the subsequent hour or more the rate of oxygen consumption was a linear function of time. With concentrations between 1.3 and 2.3 osmols per liter the full inhibitory effect took longer to develop, and in some experiments slight decreases in QO_2 occurred during the period 60 to 90 minutes after placing the tissue in the solution. With still higher concentrations the inhibitory effect was complete before the end of the 1st hour of the experiment. It is clear from the figure that hypertonicity inhibits QO_2 and that the inhibition increases with concentration up to about 2 osmols per liter; above this, further increase in concentration produces little further decrease in QO_2. No clear differences are evident in the figure in the effects of hypertonic solutions of NaCl, KCl, and sucrose. All these substances are equally inhibitory in equivalent high concentrations.

Anaerobic glycolysis of rat foot skin is also reduced in hypertonic media prepared with NaCl (Table II). In general the inhibition of $Q_G^{N_2}$ produced by

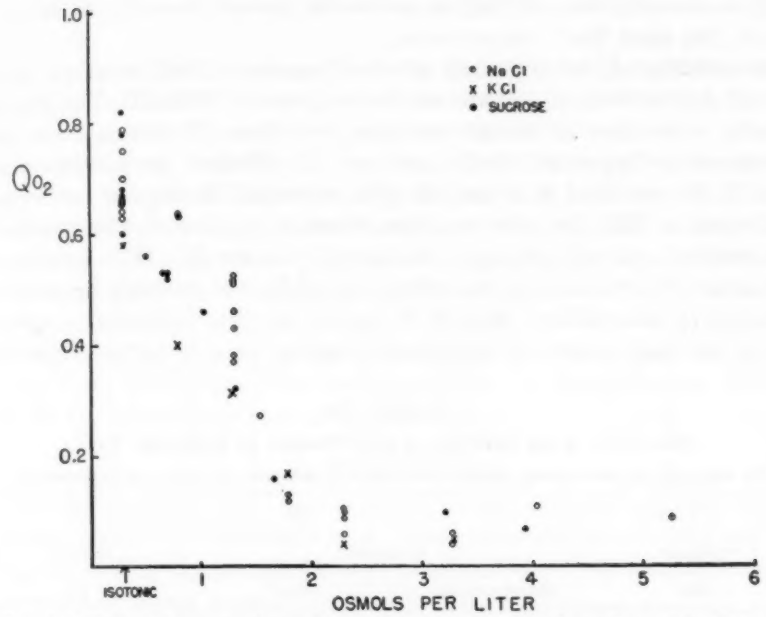

FIG. 1. Effect of hypertonic media on oxygen consumption of rat skin. The QO_2, based on initial wet weight, is shown for the period 60 to 90 minutes after placing the skin in the various media. Ringer-glucose solution was made hypertonic with either NaCl, KCl, or sucrose. See text for further details.

TABLE II

Effect of Hypertonicity on Anaerobic Glycolysis

Anaerobic glycolysis of rat foot skin in medium F (Table I) and in the same medium made hypertonic by increasing the NaCl concentration. $Q_G^{N_2}$ for 60 to 90 minutes after placing tissue in the medium.

Total concentration	No. of samples	$Q_G^{N_2}$	$Q_G^{N_2}$ Per cent of control
osmols per liter			
0.308	3	0.82	100
0.735	3	0.40	49
1.164	4	0.20	24
2.019	1	0.13	16
2.874	1	0.08	10
4.585	1	0.07	9

a given concentration of NaCl is somewhat greater than the inhibition of QO_2 in the same NaCl concentration.

Reversibility of the inhibitory effect of hypertonic NaCl solutions on the QO_2 of skin was tested, and the results are given in Table III. The QO_2 was initially determined in isotonic medium A (column I); the skin was then transferred to hypertonic NaCl media at two different concentrations (column II) for exactly 1 hour and the QO_2 determined during the last 30 minutes (column III); the skin was then rinsed in medium A, resuspended in this medium, and the QO_2 again determined (column IV). With a total concentration of 1.3 osmols per liter there was slight, but probably insignificant, evidence of reversibility. After 2.29 osmols per liter inhibition progressed during the final period in the isotonic medium. It may be concluded that

TABLE III

Reversibility of the Inhibition of QO_2 Produced by Hypertonic NaCl

The numbers in parentheses indicate numbers of samples. See text for explanation.

I	II	III	IV
Isotonic	Hypertonic		Isotonic
QO_2	Osmols per liter	QO_2	QO_2
0.64 (2) 0.65 (2)	1.3 2.29	0.49 (5) 0.08 (2)	0.51 (5) 0.05 (2)

the inhibition produced by hypertonic NaCl solutions is irreversible if the osmolarity exceeds about 1.3.

DISCUSSION

The results obtained here demonstrate that the rates of both oxygen consumption and anaerobic glycolysis of skin are reduced in hypertonic media irrespective of the substance used to produce the hypertonicity. The nature of the mechanism underlying the inhibition is, however, obscure. The respiration of avian erythrocytes is inhibited by hypertonic solutions (6). Lovelock (2) has shown that human red blood cells are hemolyzed by exposure to high concentrations of NaCl, and that much more hemolysis is produced by exposure to strong salt solutions followed by resuspension in isotonic saline. Freezing red cells at about −3°C. for 5 minutes, corresponding to a NaCl concentration of 0.8 M, was the upper limit of the temperature zone in which hemolysis occurred. Lower temperatures, corresponding to higher NaCl concentrations, produced more hemolysis (2). Fig. 1 shows that total solute concentrations of about 1.6 osmols per liter, corresponding to 0.8 M NaCl, produce about 70 per cent inhibition of the QO_2 of skin after 1 hour. Inhibition of oxygen consumption and anaerobic glycolysis in skin therefore

occurs at solute concentrations within the range calculated (2) to occur during freezing.

The nature of the damage produced by freezing red blood cells has been shown by Lovelock (7) to be, at least in part, a dispersion of a lipid or lipoprotein constituent of the cell membrane by the high electrolyte concentration. Slater and Cleland (8), however, showed that increased tonicity, obtained with sucrose, KCl, or phosphate, decreased the activity of the α-ketoglutarate oxidase system of heart muscle sarcomeres and that other oxidase systems were also inhibited by hypertonic sucrose. They interpreted their results to indicate that hypertonic solutions of these substances acted in a non-specific way on the physical state of the particles themselves. Therefore, there are at least two ways in which hypertonic solutions of electrolytes may damage cells. The relationship of hypertonicity to tissue damage in frostbite will be discussed in more detail elsewhere.

SUMMARY

The rate of oxygen consumption by rat skin is inhibited similarly by hypertonic solutions of NaCl, KCl, and sucrose. The rate of anaerobic glycolysis is inhibited by hypertonic NaCl. The inhibition of QO_2 by NaCl concentrations above about 2 osmols per liter is not reversible.

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INHIBITORY INTERACTION OF RECEPTOR UNITS IN THE EYE OF LIMULUS*

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(Received for publication, July 30, 1956)

In the lateral eye of the horseshoe crab, *Limulus*, the visual receptor units exert an inhibitory influence mutually upon one another. The discharge of impulses in any one optic nerve fiber, generated in the sensory structure of the particular ommatidium from which that fiber arises, is determined principally by the intensity of the light stimulus to the ommatidium and the state of adaptation of this receptor unit. However, the ability of an ommatidium to discharge impulses is reduced by illumination of the ommatidia in neighboring regions of the eye: its threshold to light is raised, and the frequency of the discharge that it can maintain in response to steady suprathreshold illumination is decreased. This inhibitory action is exerted reciprocally between any two ommatidia in the eye that are separated by no more than a few millimeters. As a result of inhibitory interaction among neighboring receptors, patterns of optic nerve activity are generated which are not direct copies of the patterns of external stimulation, but are modified by this integrative action that takes place in the eye itself.

These basic features of inhibition in the eye of *Limulus* have been described in detail in a recent paper (Hartline, Wagner, and Ratliff, 1956). In that paper it was shown that the anatomical basis for the inhibitory interaction is a plexus of nerve fibers lying just back of the layer of ommatidia, connecting them together. Furthermore, a direct experiment demonstrated mutual inhibitory action between two ommatidia whose respective optic nerve fibers were placed on the recording electrodes together. It is the purpose of the present paper to analyze the inhibitory interaction of receptor units in the eye of *Limulus*, and to describe quantitative properties of receptor activity that arise as a consequence of this interaction.

Method

The experiments reported in the present study are based on the measurement of the frequency of the discharge of nerve impulses from two receptor units simultane-

* This investigation was supported by a research grant (B864) from the National Institute of Neurological Diseases and Blindness, Public Health Service, and by Contract Nonr 1442(00) with the Office of Naval Research. Reproduction in whole or in part is permitted for any purpose of the United States government.

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ously, enabling the exact description of their interaction. In each experiment, a lateral eye of an adult *Limulus* was excised with 1 to 2 cm. of optic nerve and mounted in a moist chamber (maintained at 18°C.). Two small strands were dissected from the optic nerve and each placed over a pair of wick electrodes connected to its own separate amplifier and recording system. In some experiments we dissected each strand until only a single fiber remained, as evidenced by the uniformity and regularity of the action potential spikes elicited in response to illumination of the eye. In other experiments, bundles containing many active fibers were used and the isolation of single units was accomplished by coating the eye with opaque wax (a heavy suspension of lampblack in paraffin wax) and then removing the coating carefully from a very small region, exposing the corneal facet of just that one ommatidium from which it was desired to record impulses. The black wax evidently prevents internal reflections inside the cornea of the eye, for by this method perfect isolation of single units can often be obtained,—a result rarely achieved merely by focussing a small spot of light on the facet by means of a lens.

The receptors of the eye were illuminated by the same optical system that was described in the paper mentioned above. Small spots of light were projected on the eye, their sizes controlled by diaphragms and their intensities by neutral wedges. The direction of incidence of each beam could be adjusted for maximal effectiveness by a system of mirrors. A separate system was employed for each spot of light, to avoid scatter in a common optical path.

Oscillograms of the amplified action potentials in the nerve fibers were recorded photographically in some experiments. Often, however, it was preferable to measure directly the frequency with which impulses were discharged in each nerve fiber. This was done by leading the output of each amplifier through a pulse-shaper into an electronic counter. The threshold of the pulse-shaper was calibrated, and could be set to discriminate between the action potential spikes and amplifier noise with perfect reliability (in *Limulus* optic fibers, spikes can usually be obtained that are many times greater than any fluctuations of potential due to noise); the uniform output of the pulse-shaper insured perfect operation of the counting circuits. Each counter was "gated" by an electronic timer, so that only those nerve impulses occurring within a specified interval of time (usually several seconds) were registered. The gating timer was activated by a delaying timer, which permitted the counting period to be started at any desired time (usually 1 or 2 seconds) after the onset of illumination to the eye.

To obtain maximum precision in the measurement of frequency of discharge, we displayed the gating voltages and the pulses to the counter from both recording channels on a dual trace oscilloscope. We then estimated for each channel that fraction of an interval between impulses that occupied the time between the onset of the counting period and the occurrence of the first counted impulse, at the beginning of the counting period, and the corresponding fraction between the last counted impulse and the cessation of the counting period. These fractions were added to the total number of intervals registered. For greater convenience in some experiments the delaying timer, instead of activating the counter gate directly, was arranged to sensitize an electronic "trigger" which, upon the occurrence of the next nerve impulse, activated the gate to the counter. Thus the counting period always started

at the occurrence of an impulse, and only the fractional interval at the end of the gated counting period needed to be estimated. The precision gained by these methods was necessary when the counting period was short (1 or 2 seconds), and was desirable for the longer periods usually employed (7 to 10 seconds). Measurement to within about one-quarter interval was warranted by the regularity of the discharge found in many preparations, and the reproducibility of the frequencies observed.

In the experiments reported in this paper, we have confined our attention to the frequency of the discharge of impulses that is maintained at a more or less steady level during steady prolonged illumination of the eye. The transient changes in frequency that occur during the first second or two after light is turned on or off were excluded from the measurements. Exposures were fixed in duration (usually less than 10 seconds), and were repeated at fixed intervals of 2 to 5 minutes, with longer periods of rest interspersed, to minimize cumulative effects of light adaptation.

RESULTS

In the recent paper to which we have referred, direct evidence was given that receptor units in the *Limulus* eye may inhibit one another mutually. Recording the action potentials in a nerve strand containing two active fibers, it was shown that the discharge of impulses in either of the fibers was slowed when the other was brought into activity by illuminating the ommatidium from which it arose. A similar experiment is illustrated in Fig. 1, in which two strands dissected from the optic nerve, each containing a single active fiber, were placed on separate recording electrodes so that the action potentials of each of them were recorded separately. A small spot of light was centered on each of the ommatidia (designated "A" and "B") in which the fibers originated. The oscillograms show the effects of illuminating each of these small regions of the eye separately and together. The steady frequency of discharge of impulses in each fiber was less when both receptor units were active than when they were stimulated singly.

In the experiment shown in Fig. 1 the spots of light centered on the respective ommatidia were each made large enough to illuminate several receptors immediately adjacent to them in order to make the slowing of the discharges large enough to be apparent at a glance. Strictly mutual inhibition of the individual units, however, was exerted by these receptors, for similar (though less pronounced) slowing of the discharge was produced when each was illuminated by a spot so small as to be confined to the facet of its ommatidium, except for slight amounts of light that may have been scattered in the eye. In many of the experiments to be reported below, we took precautions to ensure that we were dealing with the mutual interaction of only two ommatidia by using opaque wax to effect complete optical isolation, as described in the section on Method.

From our previous study, we know that the inhibition of a receptor unit, measured by the decrease in the frequency of its discharge, is greater the

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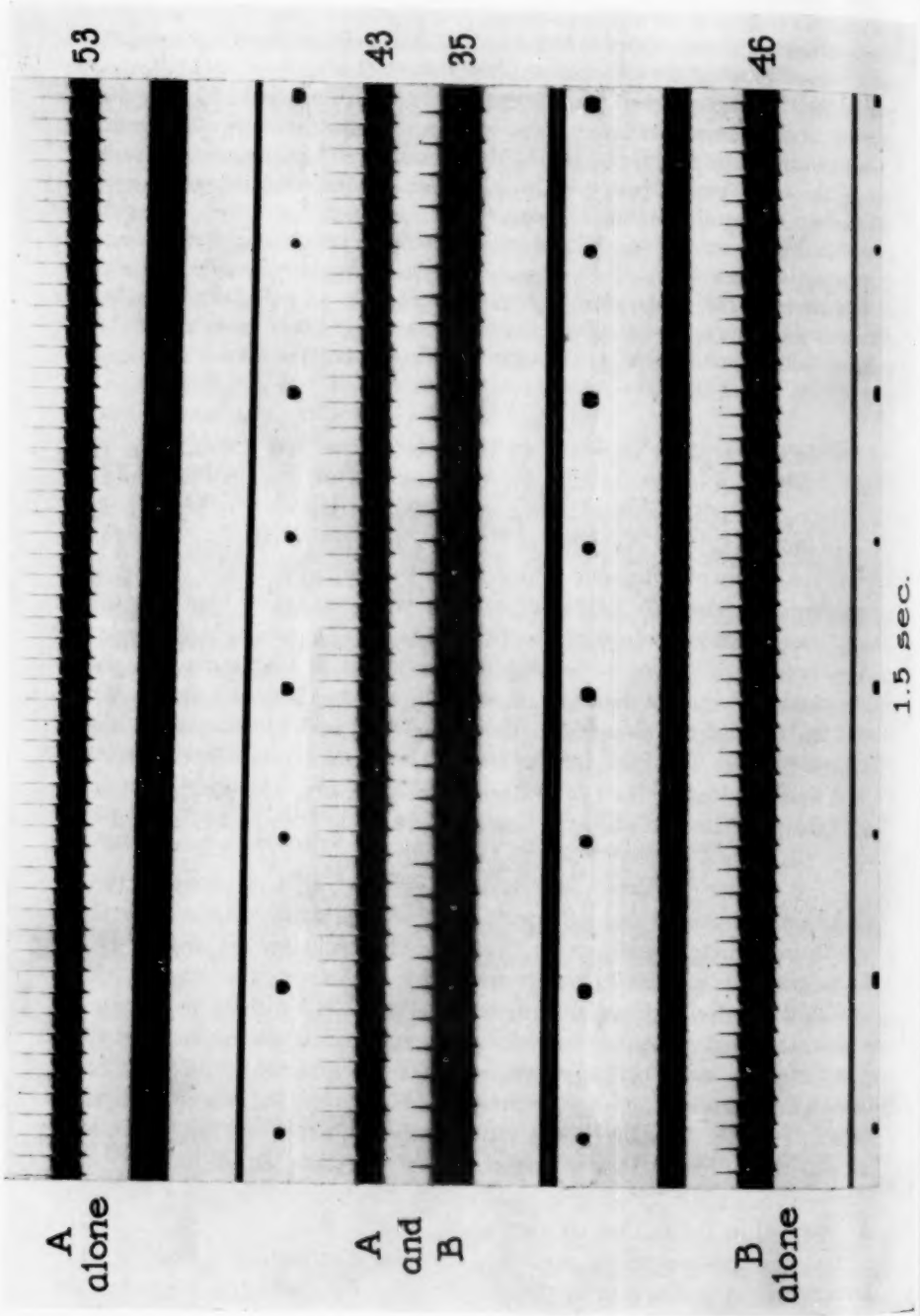
53
43
35
46
A alone
A and B
B alone
1.5 sec.

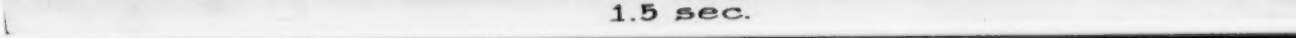

FIG. 1. Oscillograms of action potentials recorded simultaneously from two optic nerve fibers of a lateral eye of *Limulus*, showing the discharge of nerve impulses when the respective ommatidia in which these fibers originated were illuminated singly and together. In the top record, one ommatidium ("A," nerve fiber activity recorded by upper oscillographic trace) was illuminated by itself at an intensity that elicited the discharge of 53 impulses (as indicated at the right) in the period of 1.5 seconds covered by the records. In the bottom record, the other ommatidium ("B," activity recorded by lower trace) was illuminated by itself at an intensity that elicited the discharge of 46 impulses in 1.5 seconds. In the middle record, both ommatidia were illuminated together, each at the same intensity as before; ommatidium A discharged 43 impulses, ommatidium B 35 impulses, in 1.5 seconds. For A, the decrease in frequency of 10 impulses per 1.5 seconds is taken as the magnitude of the inhibition exerted upon it while B was discharging at the rate of 35 impulses per 1.5 seconds; for B, the decrease of 11 impulses per 1.5 seconds measures the inhibition exerted upon it while A was discharging at the rate of 43 impulses per 1.5 seconds. Two separate optical systems were used, each focusing a small spot of light (approximately 0.5 mm. in diameter) on the eye, one centered on ommatidium A, the other centered on ommatidium B. The spots were 1 mm. apart, center to center. Each spot illuminated about 5 ommatidia in addition to A and B. For each record, the light had been turned on 7 seconds before the start of that portion of the record shown in the figure. Time marked in one-fifth seconds; black bands above time marks are the signals of the stimulating illumination.

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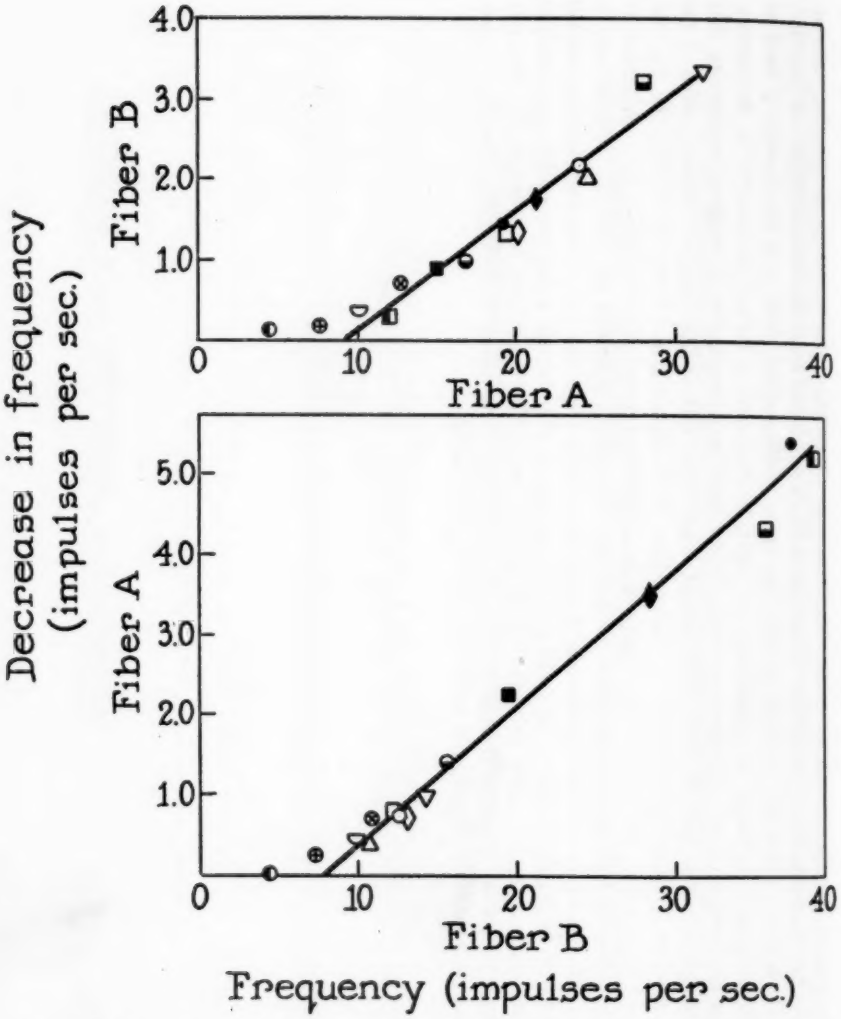

FIG. 2. Graphs showing mutual inhibition of two receptor units in the lateral eye of *Limulus*. In each graph the magnitude of the inhibition of one of the ommatidia is plotted (ordinate) as a function of the degree of concurrent activity of the other (abscissa). Sets of optic nerve fiber responses similar to those shown in Fig. 1 were analyzed as explained in the legend of that figure, each set yielding a point in the upper graph (inhibition of ommatidium B by ommatidium A) and a corresponding point (designated by the same symbol) in the lower graph (inhibition of ommatidium A by ommatidium B). The different points were obtained by using various intensities of illumination on ommatidia A and B, in various combinations.

Frequencies were determined by counting the number of impulse intervals during

stronger the stimulus to receptors that inhibit it. Experiments similar to that illustrated in Fig. 1 make it possible to show quantitatively how the amount of inhibition exerted on a receptor varies with the degree of activity of a nearby receptor unit that exerts this inhibition; the mutual interaction of two receptors can be analyzed by stimulating each of them at different intensities singly and in combination. The result of such an experiment is shown in Fig. 2. In this experiment, the frequencies of discharge of each of two ommatidia were measured, for various intensities of illumination, when each was illuminated alone and when both were illuminated together. The decrease in the frequency of discharge of each has been plotted as ordinate against the frequency of the concurrent discharge of the other as abscissa. The upper graph shows the amount of inhibition exerted upon ommatidium B by ommatidium A, as a function of the degree of activity of A; the lower graph shows the converse effect upon A of the activity of B. Both sets of points are adequately fitted by straight lines. In each case there was a fairly distinct threshold for the inhibition; each ommatidium had to be brought to a level of activity of 8 or 9 impulses per second before it began to affect the discharge of the other. Above this threshold, the frequency of discharge of B was decreased by 0.15 impulse per second for each increment of 1 impulse per second in the level of activity of A; the corresponding coefficient of the inhibitory action in the reverse direction (A acted on by B) was 0.17.

We have performed many similar experiments. Six of them, including the one just described, were done with "optical isolation," employing large nerve bundles that had exhibited activity of many fibers before the application of

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the last 5 seconds of a 7 second exposure to light (so that only the steady discharge was measured). To obtain each pair of points in the two graphs two such counts were made for each of the following conditions of illumination: A alone, B alone, A and B together, presented in an order designed to minimize systematic errors. The averages of such duplicate determinations of the magnitude of the inhibition are the values plotted in the graph. From the distribution of the differences between the individual measurements in each duplicate determination the standard error of the points in the graph was calculated to be 0.12 impulse per second. The straight lines were fitted by the method of least squares. In the upper graph the line has a slope of 0.15, which is the value of the "inhibitory coefficient" $K_{B,A}$ (effect of A on B); in the lower graph the slope is 0.17 ($= K_{A,B}$, the coefficient of the effect of B on A). The intercept of the line on the axis of abscissae is 9.3 impulses per second for the upper graph, 7.8 for the lower. Disregarding a possible "toe" at the bottom of each plot, these give the values, respectively, of the thresholds of the inhibitory effect of A acting on B, designated later in the text as r_A^0, and of B acting on A (r_B^0).

In this experiment illumination was restricted to the two ommatidia from which activity was recorded by masking the rest of the eye with opaque wax (see text). These ommatidia were 1 mm. apart.

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opaque wax to the eye to mask all but those two receptor units singled out for observation. In such experiments we could be quite certain that not any of the receptors adjacent to those under observation were excited by scattered light (since no nerve impulses from them were observed). In these experiments, therefore, the observed inhibitory effects were entirely those exerted mutually by the two receptor units upon one another. In other experiments we could be less certain about possible contributions from adjacent receptors excited by scattered light, although the scattered light was never very strong, and its effects were probably below threshold in most cases. All these experiments have shown features similar to those exhibited in Fig. 2. All showed a linear relation between the magnitude of the inhibition of one receptor (measured by the decrease in its frequency) and the degree of concurrent activity of the other (measured by its frequency). Nearly all experiments showed a "threshold" frequency below which no inhibitory effect was detected. The threshold was usually about as distinct as that shown in Fig. 2—a slight "toe" at the bottom of the curve was often noted. Although the values of the two thresholds were nearly identical in the experiment of Fig. 2, in other experiments they were not always the same for both members of an interacting pair. Likewise the slopes of the two curves often differed more than was the case in the experiment we have figured, sometimes by as much as a factor of 2.

The key to the analysis of the mutual interaction in the eye of *Limulus* lies in the correlation between the magnitude of the inhibition of a receptor and the degree of concurrent activity of the receptors that inhibit it. The degree of activity of any one of these receptors, however, depends not only on the stimulus to it but also on whatever inhibitory influences it may be subjected to in turn. It is the resultant level of activity of a receptor unit that determines the strength of the inhibition it exerts on a neighboring receptor. We have direct experimental evidence for this. Fig. 3 shows a small portion of the upper graph of Fig. 2; the points plotted as open symbols are measurements of the inhibition of ommatidium B produced by illuminating ommatidium A at two different intensities (two points at each intensity), with B illuminated at a low intensity. At the higher of the two intensities on A, which elicited a discharge in fiber A of approximately 24 impulses per second, the response of B was decreased by a little more than 2.0 impulses per second; at the lower intensity (A discharging at the rate of approximately 20 impulses per second) the discharge of B was reduced by about 1.3 impulses per second, following the trend of the solid line, which is a portion of that drawn through all the experimental points in the upper graph of Fig. 2. For these points plotted as open symbols, the activity of B itself was small (11 to 12 impulses per second); consequently the inhibition that B exerted back on A was also small (a little less than 1 impulse per second). This is

indicated by the short dotted arrows; the "tails" of these arrows are plotted at the abscissae that represent the values of the frequency obtained when ommatidium A was illuminated alone. The two points marked by the solid symbols, on the other hand, were obtained with B illuminated at higher in-

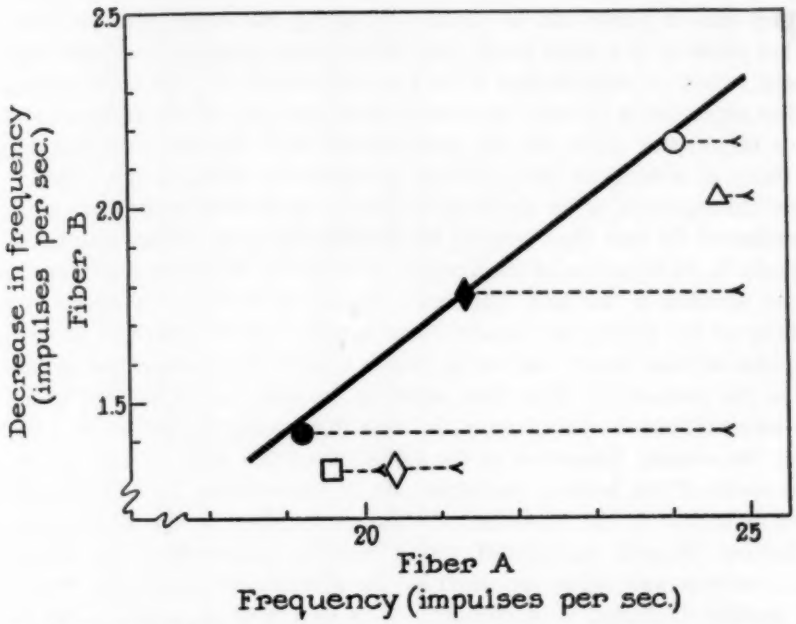

FIG. 3. Portion of the upper graph of Fig. 2. Inhibition exerted on ommatidium B is correlated with the degree of activity of ommatidium A. For the open symbols, B was illuminated at low intensity and exerted very little inhibition back on A. This is shown by the short lengths of the dotted lines, the right hand ends of which are plotted at the abscissae which give the frequency of A when it was illuminated alone. For the solid symbols, B was illuminated at high intensity and exerted strong inhibition back on A, as shown by the long lengths of the dotted lines associated with these points. For the solid symbols, ommatidium A was illuminated at the higher of the two intensities used for the open symbols. The solid line is a portion of that plotted in Fig. 2. The symbols are the same as those used for these same points in Fig. 2.

tensities. As a consequence of the resulting higher levels of activity of B (28 and 37 impulses per second), the discharge rates of ommatidium A (which for these points was illuminated at the higher of the two intensities used before) were much reduced, as can be seen by the lengths of the dotted arrows associated with these points. Corresponding to the reduced activity of A, the magnitude of the inhibition it exerted on B was smaller. This also

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followed the trend of the solid line. Thus any change in the frequency of A, whether brought about directly by changing the intensity of its stimulating light, or indirectly by changing the amount of inhibition exerted upon it as a consequence of altering the level of activity of B, resulted in comparable changes in the amount of inhibition it in turn exerted upon ommatidium B. Other sets of points can be found illustrating this same principle, both in other parts of this same graph, and in the other graph of this same experiment (effect of ommatidium B on the response of A). We have performed other experiments as well, that verify this principle for the interaction of a pair of receptor units. All the observations show that an alteration in the activity of a receptor unit, whether produced by changing the intensity of light shining on it, or by changing the inhibition exerted upon it by the other member of the pair (by changing the degree of activity of the latter), results equally in an alteration of the amount of inhibition it in turn exerts upon the other member of the pair. This result sometimes has been obscured by the scatter of the points, but usually there has been good agreement (as in Fig. 3), and we have never observed a case in which this principle was violated.

In the analysis we have just made we assumed that when the intensity on ommatidium B was increased, so that it discharged impulses at a higher rate, the ensuing diminution of the inhibition on this receptor unit was solely the result of the lowered discharge rate of ommatidium A. Our interpretation is based on the experimental finding described in the previous paper (Hartline, Wagner, and Ratliff, 1956) that the magnitude of the inhibition of a receptor unit, when measured by the absolute decrease in its frequency of impulse discharge, is independent of its own level of activity. This basic result, however, was established only as an approximation; indeed, it was noted in that paper that as the level of excitation of a "test" receptor was raised, the reduction in its frequency resulting from a fixed illumination of nearby ommatidia did in fact decrease slightly but significantly, in most experiments. This was attributed to an appreciable inhibition of the nearby ommatidia by the test receptor, just as we have done in the present case. But it might be argued alternatively that the measure of inhibition we have adopted has the inherent property that it yields a smaller value as the frequency of discharge of the test receptor is increased, and that the quantitative correlation of this measure of inhibition of one receptor unit with degree of activity of the other is only fortuitous in the present experiments. Independent experimental evidence is required to establish our interpretation more firmly.

Such independent evidence is furnished by experiments in which a third spot of light has been introduced to provide additional inhibitory influences that could be controlled independently of the two interacting receptor units whose activity was being measured. We have made use of the fact that the

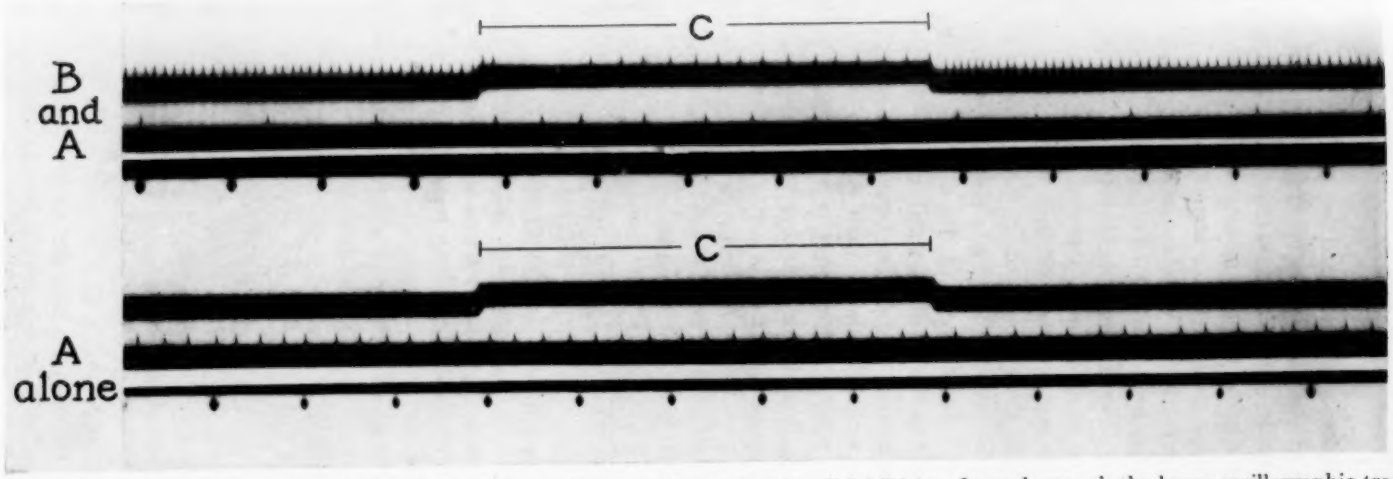

FIG. 4. Oscillograms of the electrical activity of two optic nerve fibers, showing disinhibition. In each record, the lower oscillographic trace records the discharge of impulses from ommatidium A, stimulated by a spot of light 0.1 mm. in diameter confined to its facet. The upper trace records the activity of ommatidium B, located 3 mm. from A, stimulated by a spot of light 1 mm. in diameter, centered on the facet of B, but that also illuminated approximately 8 to 10 ommatidia in addition to B. A third spot of light ("C"), 2 mm. in diameter, was directed onto a region of the eye centered 2.5 mm. from B and 5.5 mm. from A (B approximately midway between A and C); exposure of C was signalled by the upward offset of the upper trace. Lower record; activity of ommatidium A in the absence of illumination on B, showing that illumination of C had no perceptible effect under this condition. Upper record; activity of ommatidia A and B together, showing (1) lower frequency of discharge of A (as compared with lower record) resulting from activity of B, and (2) effect of illumination of C, causing a drop in the frequency of discharge of B and concomitantly an increase in the frequency of discharge of A, as A was partially released from the inhibition exerted by B.

Time marked in one-fifth seconds. The black band above the time marks is the signal of the illumination of A and B, thin when A was shining alone, thick when A and B were shining together.

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inhibitory influence becomes weaker with increased separation between an affected receptor and the region of the eye used to inhibit it (Hartline, Wagner, and Ratliff, 1956). Consequently, it is often possible to find a region on the eye that is too far from the first of the two receptor units under observation to affect that one directly by an appreciable amount, but that is near enough to the second to inhibit it markedly. We then observe the effect that the altered frequency of discharge from this inhibited receptor has on the response of the first ommatidium. Fig. 4 shows oscillograms of the activity recorded simultaneously from two receptor units, showing the effects of illuminating regions of the eye in the manner just described. When one of these receptor units (A) was illuminated alone (lower trace, lower record) its activity was not appreciably affected by illuminating a distant region of the eye (C) (signalled by the upward displacement of the upper trace). When ommatidium A was illuminated together with a small region centered on ommatidium B, which was intermediate in position between A and C, the discharge of impulses by A was markedly slower than when A was illuminated alone (lower trace, upper record). This result is attributable to the vigorous activity of ommatidium B and the receptors stimulated with it, evidenced by the discharge of impulses in B's optic nerve fiber (upper trace, upper record). Then when C was turned on, the discharge rate of A actually increased, concomitantly with a decrease in frequency of discharge from B. When C was turned off, the discharge rate of B rose again and that of A fell. We interpret this result to mean that as the receptors in the region that included ommatidium B were inhibited by illumination of region C, the decrease in their activity partially released ommatidium A from the inhibition they exerted upon it. The amount of inhibition exerted on A by region B, measured by the difference in frequency of A between the lower record (A alone) and the upper (A with B) was less when C was being illuminated than when it was not; this diminished inhibition paralleled the lessened degree of activity recorded in fiber B.

The parallelism between the degree of activity of a receptor subjected to inhibition and the inhibition it in turn exerts on its neighbors is quantitative. This is shown in Fig. 5, drawn from data obtained from the same experiment as Fig. 4, except that the spot of light centered on ommatidium B was reduced in size, so that it was confined to that ommatidium. Consequently, the inhibition exerted by B may be correlated strictly with the activity recorded in its axon. In Fig. 5, the inhibition (decrease in frequency) of ommatidium A is plotted as a function of the frequency of discharge of ommatidium B; the open symbols are for two different values of light intensity on ommatidium B with no illumination on region C. The solid symbols are for a high intensity on B, but with the addition of light on the region C. The effect that C had on the discharge of B is represented by the length of the

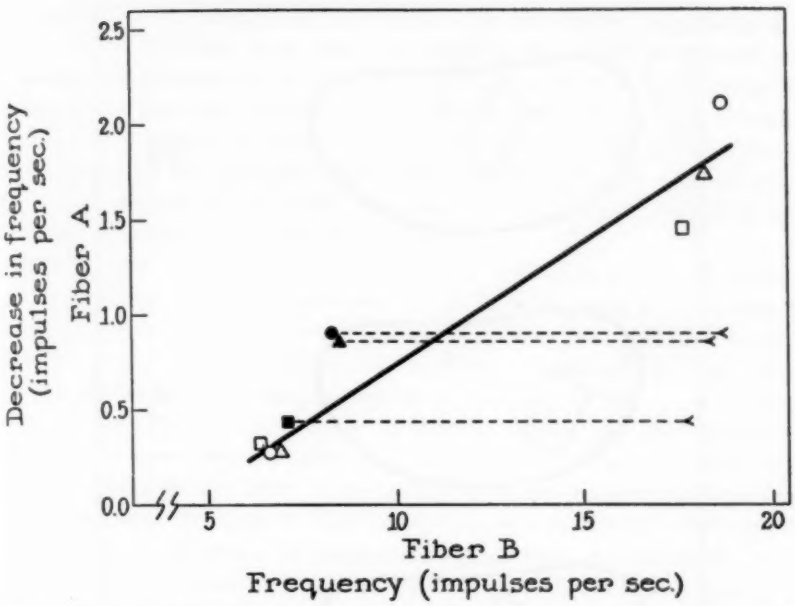

FIG. 5. Decrease of the inhibition exerted on one receptor unit (A) by another (B), as a result of inhibiting the activity of the second by illuminating a region of the eye (C) close to it. From the same experiment as that of Fig. 4 (see legend), but with the spot of light on ommatidium B reduced to 0.2 mm. diameter. The inhibition of ommatidium A was measured by the difference between its frequency when illuminated alone and when it was illuminated together with ommatidium B; this has been plotted as ordinate against the frequency of B as abscissa (as in Figs. 2 and 3). Three points were determined for a high intensity and three for a low intensity on B, when there was no illumination on C (open symbols). Three points were similarly determined for a high intensity on B when the nearby region of the eye, C, was illuminated (see legend of Fig. 4). These points are designated by the solid symbols. The lengths of the dotted lines associated with these points show the amount of reduction in the frequency of discharge of ommatidium B, as a result of the inhibition exerted upon it by C. Corresponding to this reduction in the activity of B, the inhibitory effect it in turn exerted on A was reduced, by an amount that is in quantitative agreement with the reduction obtained by lowering the intensity on B, as given by the solid line drawn through the open symbols. Illumination of the region C with no light shining on ommatidium B had very little effect on the activity of A: a reduction in frequency of 0.3 impulse per second was the maximum observed (the region C must have contributed even less than this amount to the total inhibition, since the receptors in it were also subject to inhibition by the activity of B).

Determination of the frequencies was made as described in the legend of Fig. 2.

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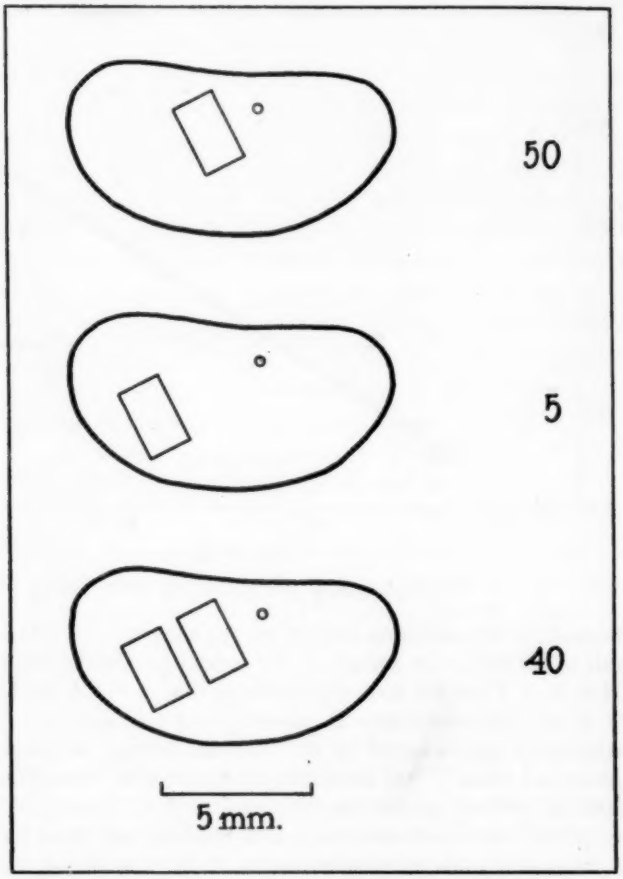

FIG. 6. A schematic diagram of patterns of light on a lateral eye of *Limulus* in an experiment illustrating disinhibition. The heavy lines are sketches of the outer margins of the eye. A small spot of light marked "o" was centered on the facet of a "test" ommatidium whose activity was measured by recording the action potentials in the optic nerve fiber arising from it. This spot was small enough to illuminate only the ommatidium on which it was centered. For each measurement the small spot of illumination was turned on for 12 seconds at a constant intensity. The number of impulses discharged by the test ommatidium in the last 10 seconds of such exposure was determined when the ommatidium was illuminated alone, and again when it was illuminated together with rectangular patches of light on other regions of the eye, as shown in the three sketches. The difference in the counts (decrease in frequency, in impulses per 10 seconds) measures the magnitude of the inhibition exerted on the test ommatidium by the receptors in the regions illuminated by the rectangular patches of light; these differences are given at the right for the respective parts of the experiment. Upper sketch, a rectangular patch of light near the test omma-

dotted lines. This experiment shows that the diminution in activity of ommatidium B had the same effect in reducing the inhibition exerted on A, whether that diminution was the result of inhibition of B by illumination of region C, or the result of reduction in the intensity of the light stimulus to B. Thus the degree of activity of a receptor unit does indeed determine, quantitatively, the strength of the inhibition it exerts on another receptor unit. Our analysis of the interaction between two receptor units illuminated together is therefore substantiated.

The release of a receptor unit from the inhibiting effects of others, by causing those others to be inhibited by yet a third group of receptors, is interesting physiologically. Such "disinhibition" is not difficult to obtain, though it may require some pains to show a strong effect. We have performed one other experiment similar to that of Figs. 4 and 5, recording from two fibers and using a third spot of light to inhibit one and disinhibit the other. It is considerably easier to show disinhibition when recording from only one receptor, for then it is possible to choose a favorable combination of locations for the spots of light that serve to inhibit and to disinhibit this test receptor. We have done many such experiments. An example is given in Fig. 6; the experiment is explained in its legend. Instead of focussing spots of light in various locations on the surface of the eye, disinhibition can also be demonstrated by using sources of light in various places in the external visual field, where the directional sensitivity of the ommatidia determines the location in the eye of the groups of receptors stimulated by the respective light sources. Dr. William Miller, in our laboratory, has also demonstrated disinhibition of receptors in the median eye of *Limulus* (a simple eye), using light sources in the external visual field.

Disinhibition simulates facilitation: illumination of a distant region of the eye results in an increase in the activity of the test receptor. In the *Limulus* eye the dependence of such an effect on the stimulation of receptors in an intermediate region of the eye (to produce the original inhibition) makes it

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tidium produced a decrement of 50 impulses in 10 seconds. Middle sketch, a similar rectangular patch of light farther away from the test ommatidium produced a decrement of 5 impulses in 10 seconds. Lower sketch, both patches of light shining together produced a decrement of only 40 impulses in 10 seconds.

Thus in the last case the distant patch, rather than adding to the inhibition exerted by the near one, caused a disinhibition of 10 impulses per 10 seconds. As established by the experiments of Figs. 4 and 5, this was the result of the inhibition of the receptors in the near patch by the activity of those in the distant one, with the consequence that they in turn exerted less inhibition on the test ommatidium. This release of the test ommatidium from the inhibition exerted by the receptors in the near patch was greater than the slight inhibitory effect exerted directly on the test ommatidium by the receptors in the distant patch.

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easy to recognize the mechanism involved. But if such a group of intermediate inhibiting elements were active spontaneously, or through uncontrolled influences, it might be difficult to recognize the true nature of a disinhibiting action. Perhaps the most significant aspect of these experiments showing disinhibition is the principle that they reveal, that indirect effects may extend considerably beyond the limit of the direct inhibitory connections among the receptors of the eye. Indeed, no member of the population of receptors is completely independent, under every condition, of the activity in any part of the eye. This is a direct consequence of the principle of interaction that we have established: the inhibiting influence exerted by a receptor depends on its activity, which is the resultant of the excitatory stimulus to it and whatever inhibitory influences may in turn be exerted upon it.

The principles that we have established experimentally may be conveniently summarized in a simple algebraic expression. The activity of a receptor unit—its response (r)—is to be measured in the present case by the frequency of the discharge of impulses in its axon. This response is determined by the excitation (e) supplied by the external stimulus to the receptor, diminished by whatever inhibitory influences may be acting upon the receptor as a result of the activity of neighboring receptors. The excitation of a given receptor is to be measured by its response when it is illuminated by itself, thus lumping together the physical parameters of the stimulus and the characteristics of the photoexcitatory mechanism of the receptor. Each of two interacting receptor units inhibits the other to a degree that depends (linearly) on its own activity. The responses of two such units are therefore given by a pair of simultaneous equations:

$$r_A = e_A - K_{A,B}(r_B - r_B^0)$$

$$r_B = e_B - K_{B,A}(r_A - r_A^0)$$

in which the subscripts are used to label the respective receptor units. In each of these equations, the magnitude of the inhibitory influence is given by the last term, written in accordance with the experimental findings as a simple linear expression. The "threshold" frequency that must be exceeded before a receptor can exert any inhibition is represented by r^0. The "inhibitory coefficient," K, in each equation is labelled to identify the direction of the action: $K_{A,B}$ is the coefficient of the action of receptor B on receptor A; $K_{B,A}$ *vice versa*. It is to be clearly understood that the equations do not apply in the ranges of responses for $r < r^0$ in either case: negative values of inhibition must be excluded since they are never observed. Also, r and e, by their nature (being measured by frequencies), cannot be negative. Appropriate changes must be made in the equations in those ranges of the variables where these restrictions apply: if, for example, $r_B < r_B^0$, the first equation must be replaced by $r_A = e_A$; if, to choose another example, r_B is

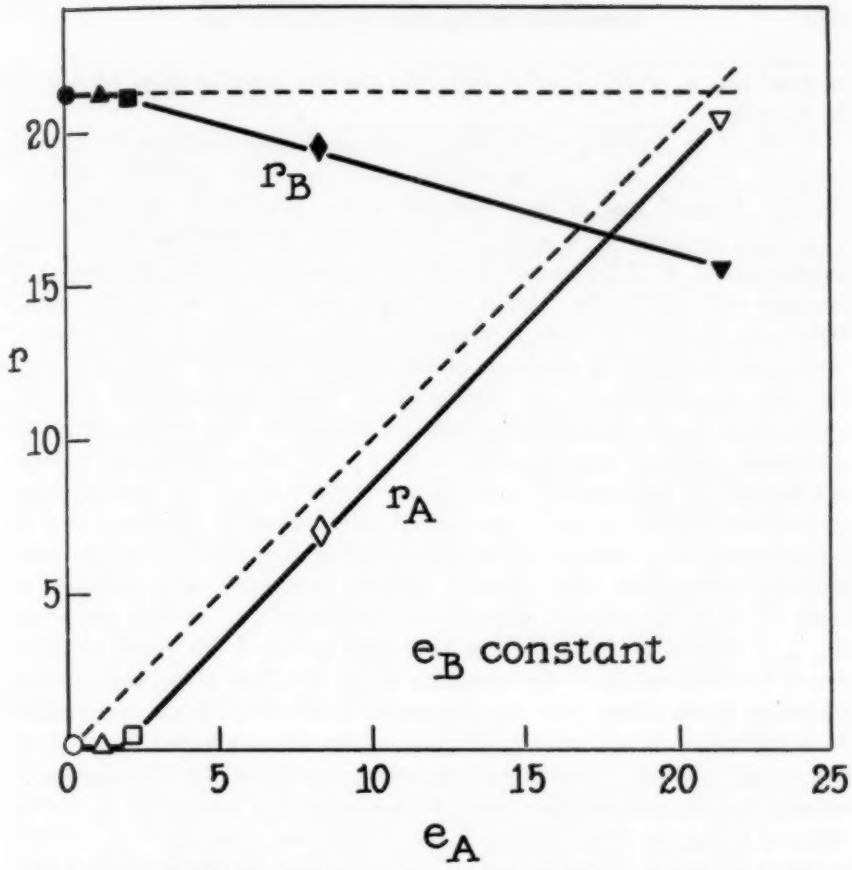

FIG. 7. Graph showing the relation of the responses of two interacting receptor units. One ommatidium (B) was illuminated at a fixed intensity; the intensity on the other (A) was varied. Responses (r) were measured by the frequency of the steady discharge of nerve impulses (last 7 seconds of a 10 second exposure to light). When both A and B were illuminated together, r_A refers to the response of ommatidium A (open symbols), r_B refers to the response of ommatidium B (solid symbols). The excitation of A, designated by e_A, is measured by the response of this ommatidium when it was illuminated alone; the excitation of B, designated by e_B, is measured by the response of B when it was illuminated alone. For each set of exposures (A alone, B alone, A and B together) with a given intensity for ommatidium A, the values of r_A and r_B obtained have been plotted (ordinates) against the value of e_A (abscissa). Values obtained for e_B were consistent within 0.1 impulse per second for all the sets of exposures; their average (21.2 impulses per second, shown by the horizontal dotted line) has been used in the calculations. The solid lines are the solutions of the simultaneous equations given in the text; the calculations and values of the constants are given there. The vertical distance from each dotted line ($r = e_A$, and $r = e_B$) to the corresponding solid line beneath it shows the amount of the inhibition for each receptor at that value of e_A.

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so great that $e_A < K_{A,B}(r_B - r_B^0)$, then the first equation must be replaced by $r_A = 0$.

In Fig. 7 we give the results of an experiment using two receptor units to illustrate the solutions of the simultaneous equations governing their responses. A small spot of light centered on ommatidium B was maintained at a fixed intensity, such as to give a frequency of discharge (when it was shining alone) of 21.2 impulses per second in the optic nerve fiber from this receptor unit. This is the value of e_B. Another small spot, centered on ommatidium A, was set at various intensities of illumination; at each intensity the steady frequency of discharge of this receptor unit was determined when the spot illuminating it was shining alone. These measurements give the value of e_A, for the corresponding stimulus intensities. The frequencies of the discharges obtained when the two receptor units were illuminated together are plotted for each one (r_A and r_B) as functions of e_A. On another graph (not shown) similar to Fig. 2 we plotted the decrease in frequency ($e - r$) for each unit as a function of the corresponding frequency (r) of the other, obtaining plots which, when fitted by straight lines, gave values of the intercepts $r_A^0 = 0$ (an unusual value, in our experience), $r_B^0 = 4.0$, and slopes $K_{A,B} = 0.09$, $K_{B,A} = 0.26$. The solid lines of Fig. 7 are drawn as determined by these values of the constants in the solutions of the simultaneous equations given above. For low intensities of illumination on ommatidium A (small values of e_A), activity of this receptor was prevented ($r_A = 0$) by the strong inhibition exerted by the activity produced by illumination of ommatidium B, and therefore, since no inhibition was exerted on B, the activity of B was the same as when it was illuminated alone ($r_B = e_B$). At the intensity for which the excitation e_A just overcame the effects of the inhibition exerted by B ($e_A = K_{A,B}[e_B - r_B^0] = 1.6$), receptor A began to respond; as e_A increased, the frequency of its discharge increased linearly with a slope of $1.024 \left(= \frac{1}{1 - K_{A,B} K_{B,A}} \right)$. At this same value of e_A (since $r_A^0 = 0$), the inhibition by A on B began to be exerted, and r_B decreased linearly with increasing e_A, the slope being $-0.27 \left(= \frac{-K_{B,A}}{1 - K_{A,B} K_{B,A}} \right)$. (The dotted lines show the form the graphs would have taken had there been no inhibition.) In this experiment each spot of light actually illuminated about 8 or 9 other ommatidia in the immediate vicinity of the one on which it was centered and from which activity was recorded. For purposes of illustration, it is permissible to treat the results as though individual units were interacting, although in actuality it was each small group. That the principles involved hold rigorously when only two single receptor units are actually used is inherent in the treatment, for these principles were derived from the experi-

ment of Fig. 2 (and those like it), in which strict optical isolation of individual ommatidia was employed.

The mutual interdependence of two receptor units responding to steady illumination is thus concisely and accurately described by a pair of simultaneous equations. Similar equations hold for the responses of any two ommatidia that are close enough together in the eye to interact. When more than two interacting elements are activated, similar relations apply simultaneously to the responses of all of them. In addition, however, each receptor unit is subjected to inhibitory influences from all the others, and the degree to which its response is decreased is known to be greater, the greater the number of neighboring ommatidia that are stimulated (Hartline, Wagner, and Ratliff, 1956). The simultaneous equations governing the responses of more than two ommatidia therefore must contain terms expressing the inhibition contributed from all the active elements, combined according to some law of spatial summation. Experiment shows that simple arithmetic addition of such terms is adequate to describe spatial summation of inhibitory influences in the eye of *Limulus*. In a paper that will follow (see also Hartline and Ratliff, 1954 and Ratliff and Hartline, 1956) we will describe the experiments that establish this law of spatial summation and will illustrate some of the effects that are obtained when more than two receptor units in the eye inhibit one another mutually.

SUMMARY

The inhibition that is exerted mutually among the receptor units (ommatidia) in the lateral eye of *Limulus* has been analyzed by recording oscillographically the discharge of nerve impulses in single optic nerve fibers. The discharges from two ommatidia were recorded simultaneously by connecting the bundles containing their optic nerve fibers to separate amplifiers and recording systems. Ommatidia were chosen that were separated by no more than a few millimeters in the eye; they were illuminated independently by separate optical systems.

The frequency of the maintained discharge of impulses from each of two ommatidia illuminated steadily is lower when both are illuminated together than when each is illuminated by itself. When only two ommatidia are illuminated, the magnitude of the inhibition of each one depends only on the degree of activity of the other; the activity of each, in turn, is the resultant of the excitation from its respective light stimulus and the inhibition exerted on it by the other.

When additional receptors are illuminated in the vicinity of an interacting pair too far from one ommatidium to affect it directly, but near enough to the second to inhibit it, the frequency of discharge of the first increases as it is partially released from the inhibition exerted on it by the second (disinhibition).

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Disinhibition simulates facilitation; it is an example of indirect effects of interaction taking place over greater distances in the eye than are covered by direct inhibitory interconnections.

When only two interacting ommatidia are illuminated, the inhibition exerted on each (decrease of its frequency of discharge) is a linear function of the degree of activity (frequency of discharge) of the other. Below a certain frequency (often different for different receptors) no inhibition is exerted by a receptor. Above this threshold, the rate of increase of inhibition of one receptor with increasing frequency of discharge of the other is constant, and may be at least as high as 0.2 impulse inhibited in one receptor per impulse discharged by the other. For a given pair of interacting receptors, the inhibitory coefficients are not always the same in the two directions of action. The responses to steady illumination of two receptor units that inhibit each other mutually are described quantitatively by two simultaneous linear equations that express concisely all the features discussed above. These equations may be extended and their number supplemented to describe the responses of more than two interacting elements.

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THE ROLE OF A CONTAMINANT IN THROMBIN IN THE HUMAN PLASMIN ASSAY SYSTEM*

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(Received for publication, June 18, 1956)

In recent years there has been increasing interest in the use of plasminogen as a potential therapeutic agent in the treatment of thrombotic disorders. Much work has been done on the isolation (1–5), mechanism of activation (6–9), characterization (10), and development of assay methods (11–13), for the components of the fibrinolytic system. Two assay methods are generally used. One measures the proteolytic activity of the active enzyme on a casein substrate; the other the lysis of a fibrin clot produced with a standard amount of bovine fibrinogen. Unfortunately bovine fibrinogen is reported to be contaminated with bovine plasminogen (14), and interpretation of assay results always requires examination of the role this contaminant could have played in determining the rate of clot lysis. A "human plasminogen" preparation contains two, at this time, inseparable substances—proactivator and proenzyme (plasminogen). The best known activator is streptokinase. Streptokinase converts proactivator to activator, which in turn converts the proenzyme plasminogen to the active enzyme plasmin. Plasmin can then be assayed by the proteolytic or fibrinolytic method. The rate of casein digestion is relatively low, but a tremendous amount of fibrin can be lysed by the enzyme in a very short time. The high fibrinolytic activity has been thought to be due to activation by the human activator of the bovine plasminogen contaminating the bovine fibrinogen substrate (14). Our calculations have revealed that the amount of bovine plasminogen that would have to be present as a contaminant to account for this activity is greater than the amount of bovine fibrinogen present in the assay system. In addition, there are reports of the great

* This investigation was supported by a grant (C2009) from the United States Public Health Service.

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fibrinolytic activity of streptokinase-activated human plasmin, coupled with a low fibrinogenolytic activity, whereas bovine plasmin exhibits comparable fibrinolytic and fibrinogenolytic activity (15). Some authors have attributed the difference in fibrinolytic and fibrinogenolytic activity to a potentiation of human plasmin activation in the clotting process (16). These puzzling and contradictory reports in the literature on any measurement when clotting has occurred prompted our investigation of the role of thrombin in the plasmin assay systems. It was soon apparent that the thrombin preparation was a key factor in these anomalous results, and we are reporting here the observations which led to this conclusion. Moreover, we have isolated from bovine thrombin preparations the factor responsible for the confusion and have shown it to be similar in physical and enzymatic properties to bovine plasminogen.

Materials and Methods

Fibrinopeptide.—One ml. of normal oxalated human plasma was diluted to 10 ml. with water, 2 ml. of 0.1 M $CaCl_2$ was added, and the mixture clotted with 0.2 ml. (100 N.I.H. units) of thrombin. After 30 minutes the clot was spun out on a glass rod and the supernate placed in a 23/32 inch Visking sausage casing. The casing was immersed in 5 ml. of phosphate buffer (0.1 M, pH 7.4) in a 20 ml. vial and rocked at 4° for 24 hours. The dialysate should contain the same concentration of the dialyzable fibrinopeptide as the contents of the dialyzed bag.

Thrombin. –Parke, Davis and Co., Detroit, thrombin topical was used in all assays unless otherwise stated. A highly purified thrombin prepared by Dr. Walter H. Seegers was used in a single assay, as was a commercial Upjohn preparation. The commercial preparations were dissolved at a concentration of 500 units/ml. of saline. One mg. of the purified Seegers preparation was dissolved in 1 ml. of saline.

Bovine Fibrinogen.—(Armour and Company Laboratories, Chicago). A 1 per cent solution in veronal buffer was used in the fibrinolytic assays.

Veronal Buffer.—4.12 gm. of sodium barbital per liter adjusted to pH 7.4 with sodium hydroxide.

Streptokinase (*Varidase.*—Lederle Laboratories, Pearl River, New York).—100,000 units streptokinase and 25,000 units streptodornase were dissolved in 10 ml. of saline.

Bovine Plasminogen.—Bovine plasminogen was obtained through the courtesy of Dr. E. Loomis of Parke, Davis and Co.

Human Plasminogen.—Human plasminogen prepared by a modification (17) of the Kline method (18) was obtained from the Merck, Sharp and Dohme Research Laboratories, West Point, Pennsylvania. 35 mg. was dissolved in 95 ml. of water and adjusted to neutral pH by the rapid addition of 5 ml. of 2 M phosphate buffer.

Human Fibrinogen.—Human fibrinogen was prepared from a pool of oxalated normal human plasma by the method of Ware, Guest, and Seegers (19).

Casein.—A 1.8 per cent solution of casein in pH 7.4 phosphate buffer was prepared by the method of Mullertz (6), and stored frozen in small bottles.

Potato Starch.—Powdered Maine potato starch was freed of ultraviolet-adsorbing impurities by washing each pound with 1 liter of 0.01 M acetic acid at 50°, removing the acid by many distilled water washes, washing with phosphate buffer, water, alcohol, ether, and air drying.

Radioiodine.—Iodine-131 was obtained through the generosity of the Oak Ridge Laboratories of the Atomic Energy Commission. 0.5 mc. of the carrier-free isotope was used to label the proteolytic enhancing factor isolated from commercial thrombin.

Methods

Fibrinolytic Assay.—The method previously reported (2) has been used. In the determination of the effect of fibrinopeptide on fibrinolytic activity, 0.4 ml. of the fibrinopeptide dialysate was added to a mixture of 0.035 mg. of "human plasminogen," 1000 units of streptokinase (SK), 0.15 ml. of 1 per cent bovine plasminogen, and 0.15 ml. of veronal buffer. In assays to determine the effect of the amount of thrombin, the fibrinopeptide was replaced by 0.4 ml. of veronal buffer. In all assays the volume of thrombin solution used for clotting was 0.1 ml., and the amount of thrombin used was varied by dilutions with veronal buffer. In assays in which human fibrinogen was used as a substrate, 0.15 ml. was used. In determining the "human plasminogen"-lysis time curve of Fig. 5, 1000 units of streptokinase were placed in each tube and varying dilutions of plasminogen solution added for a 3 minute activation period prior to the addition of 0.15 ml. of bovine fibrinogen and sufficient veronal buffer to bring the volume to 0.9 ml. Clotting was accomplished with 50 units of thrombin.

Proteolytic Assay.—The proteolytic assay method was a previously reported variation (7) of the method of Mullertz (6). When thrombin was present in the assay system, it was added just prior to the addition of the final reactant, casein. Since activation of plasminogen preparations with streptokinase is very rapid, there is no effect of varying the order of addition of the reagents. In the determination of proteolytic activity in the presence of thrombin in which streptokinase concentration was varied, 3.0 ml. of casein solution was added to 3.0 ml. of a phosphate buffer solution containing 250 units of thrombin, 0.52 mg. of "human plasminogen," and 0 to 5000 units of streptokinase. The mixtures were incubated for 4 hours, and the results are reported as the γ of tyrosine liberated in this time (Fig. 1). The proteolytic enhancing activity of the starch electrophoresis eluates were determined similarly. Either 2 ml. of each eluate was added to 1 ml. of phosphate buffer, or 1 ml. of eluate was added to 2 ml. of buffer containing 0.35 mg. of "human plasminogen" and 150 units of streptokinase. After the addition of 3.0 ml. of casein 2 minute and 4 hour samples were assayed for tyrosine. Spontaneous proteolytic activities were also determined on the eluates by a similar assay replacing the 1 ml. of plasminogen and streptokinase by 1 ml. of phosphate buffer.

In the experiment in which proteolytic activity was measured in the absence and presence of bovine fibrinogen, the same amount of "human plasminogen" was used as above, and either 1.5 ml. of phosphate buffer or 1.5 ml. of 1 per cent fibrinogen was added. The "human plasminogen" was pre-activated with 0 to 3600 units of

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streptokinase/mg., and a 0.1 ml. portion of the activated mixture was used for fibrinolytic determination. (Fig. 4)

Starch Electrophoresis.—Starch electrophoresis of the thrombin preparation and of the bovine plasminogen was carried out for 17 hours at 4° on a 40 cm. starch block of 1 cm. thickness and 19 cm. width. A pH 7.6 phosphate buffer $\left(\frac{\Gamma}{2} = 0.1\right)$ was used with filter paper strips connecting the starch block to the electrode vessels. Approximately 50 ma. were drawn at a potential difference between the platinum electrodes of 400 volts. 5000 units of thrombin were dissolved in 3 ml. of water and dialyzed for 2 hours against a portion of the electrophoresis buffer. A 0.5 cm. section was cut out of the center of the starch block and the hole filled with a dry paste of the dialyzed thrombin solution and starch. The starch block was allowed to equilibrate for 1 hour with the buffer in the electrode vessels before the current was turned on. At the completion of electrophoresis the block was cut into sections of 1 cm. width and the protein eluted by transferring the section to a sintered glass funnel and stirring with 5 ml. of saline. The concentration of protein eluted from each centimeter section of the starch block was estimated by measuring the optical density at 275 mμ of the eluate in 1 cm. cuvettes in a Beckman spectrophotometer. Other assays were then carried out on the eluate. Starch electrophoresis of 60 mg. of bovine plasminogen and of a mixture of 30 mg. of bovine plasminogen and the radioiodinated plasmin-enhancing factor from thrombin were carried out similarly. Radioactivity was measured in a γ well counter using a filter which diminished activity by a factor of 32.8.

Clotting Time.—A 0.1 ml. portion of the starch electrophoresis eluates was blown into a solution of 0.15 ml. bovine fibrinogen in 0.75 ml. of veronal buffer in a 13 × 100 mm. test tube, and the tube gently tilted until clotting occurred.

Radioiodination of the Thrombin Contaminant.—The starch electrophoresis eluate containing the proteolytic activity enhancing factor was lyophilized and iodinated with I^{131} according to the method of Pressman and Eisen (20).

RESULTS AND DISCUSSION

Fibrinopeptide.—If one is to accept the view of Bastian and Hill (16) that the process of clotting accelerated the activation of plasminogen with streptokinase, then some substance formed during the clotting process must be responsible for this acceleration. The peptide separated from fibrinogen by the action of thrombin might reasonably be expected to be that substance. When fibrinopeptide dialysate was added to the fibrinolytic assay system containing human plasminogen and streptokinase, and the mixture clotted with a 1/50 thrombin dilution (1 unit); a lysis time of 8 minutes was observed. In the absence of the fibrinopeptide the clot also lysed in 8 minutes. The presence of the additional fibrinopeptide therefore had no effect on the rate of lysis. However, if the same mixture was clotted with undiluted thrombin (50 units) a lysis time of 2.5 minutes was observed. Since the fibrinopeptide is obtained from the breakdown of fibrinogen and the fibrinogen content of the two assay

systems remains unchanged, it is difficult to see how more fibrinopeptide would be formed in the presence of excess thrombin or indeed how fibrinopeptide could be involved.

Fibrinolytic Activity of Fibrinolytic Systems Clotted with Varying Concentrations of Thrombin.—The discordant lytic activities observed above at two different thrombin levels suggested that thrombin concentration might have a determining role in fibrinolytic assay. Five different thrombin concentrations were used for clotting a system containing "human plasminogen," streptokinase, and fibrinogen. The results are shown in Table I. High thrombin concentrations yielded rapid lysis, low thrombin concentrations gave slow lysis. The more thrombin used in clotting the more fibrinolytic enzyme appeared to be present to lyse the clot. This was the first positive evidence that it was not the clotting process itself, but rather some property of the thrombin preparation that was responsible for the acceleration of lysis.

TABLE I

Fibrinolytic Activity of Plasmin Clotted with Varying Amounts of Thrombin

Units of thrombin	Lysis time
	min.
50	2.5
5	5
2	7.5
1	8
0.5	10.5

TABLE II

Proteolytic Activity of Plasmin

	$\frac{\gamma \text{ tyrosine}}{4 \text{ hours}}$
(1) 250 units thrombin	225
(2) 0.52 mg. "human plasminogen" plus 1000 units SK	304
(3) 250 units thrombin and 0.52 mg. "human plasminogen" and 1000 units SK	1360
(4) 250 units thrombin and 0.52 mg. "human plasminogen"	235
(5) 0.52 mg. human plasminogen	10
(6) 1000 units SK	2

Seegers' highly purified thrombin at concentrations of 0.1, 0.01, and 0.001 per cent were used to clot the same mixture of human plasminogen, streptokinase, and bovine fibrinogen. Lysis times of 9, 9, and 10 minutes respectively were observed. The purified thrombin clotted this mixture as rapidly as the Parke, Davis preparation yet it failed to produce the same rapid lysis. Since

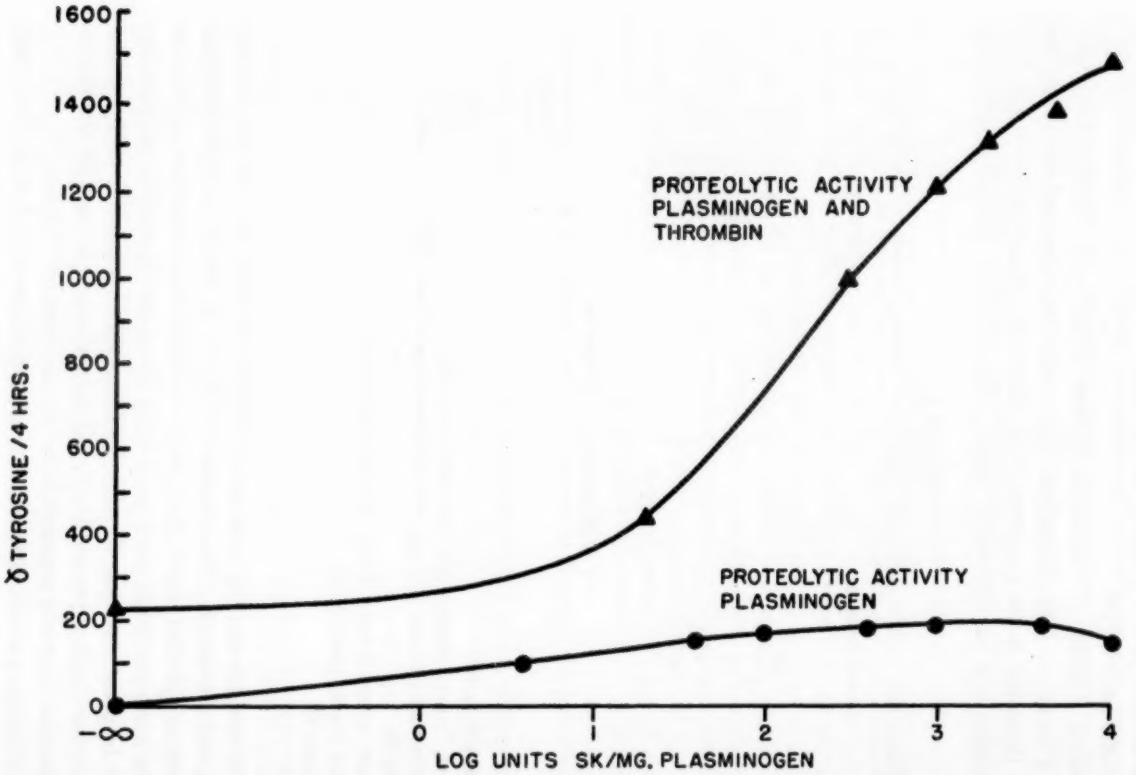

FIG. 1. Increase in proteolytic activity of plasminogen and plasminogen plus thrombin in the presence of increasing amounts of streptokinase (SK). The results are expressed as the γ of tyrosine liberated in 4 hours from a 0.9 per cent casein substrate by 0.52 mg. plasminogen in the presence and absence of 250 units of commercial bovine thrombin.

the high thrombin concentration was not responsible for the increase in lysis rate, then there must be some component in the commercial thrombin preparation which is not thrombin, and which can contribute to plasmin activity.

Fibrinolytic Activity of Human Fibrinogen and Streptokinase Clotted with Thrombin.—Human fibrinogen containing added streptokinase and clotted with 1 unit of thrombin required more than 2 hours for lysis. When clotted with 50 units of thrombin, lysis occurred in 15 minutes. Human fibrinogen prepared by the method of Ware, Guest, and Seegers under the action of streptokinase contains no fibrinogenolytic activity. It is therefore free of both proactivator and proenzyme together but it may contain one or the other. When 1 unit of thrombin is added for clotting no lysis occurs. However, 50 units of thrombin supplies sufficient quantity of one of the fibrinolytic components missing from the human fibrinogen for lysis to occur in 15 minutes. If human fibrinogen contains proactivator, then the proenzyme is supplied by the high concentration of thrombin. If the human fibrinogen contains proenzyme, then the high concentration of thrombin must supply proactivator. However, the latter possibility must be ruled out since the thrombin is of bovine origin and bovine proactivator is not converted to activator by streptokinase.

Proteolytic Activity of Human Plasminogen and Streptokinase in the Presence of Thrombin.—The tyrosine produced in 4 hours by the action of various enzyme and enzyme mixtures is reported in Table II. The sum of the tyrosine liberated by (*a*) thrombin alone, and by (*b*) human plasminogen together with streptokinase is far less than that liberated by (*c*) a mixture of thrombin, human plasminogen, and streptokinase. In this system which contained no fibrinogen and in which no clotting has occurred, there was a marked enhancement of the proteolytic activity of plasmin in the presence of thrombin. These results were of such great interest that the experiment reported in Fig. 1 was set up. Less than 30 units of streptokinase activated enough of the human proactivator to convert all of the human proenzyme to plasmin, and in the absence of thrombin a maximum plateau in the proteolytic activity was observed. Further additions of streptokinase could bring about no increase in proteolytic activity since all the proenzyme had already been converted to active plasmin. In the proteolytic assay to which thrombin had also been added, proteolytic activity increased as streptokinase concentration increased, and was still increasing at 5000 units of streptokinase, a level at which inhibition begins to appear in the absence of thrombin (21).

Since the human plasmin content of the proteolytic system remains constant after the addition of 30 units of streptokinase, there must be some other substance present which can combine with increasing amounts of streptokinase and bring about the increased activity observed in the presence of the thrombin preparation. In a previous report (7) it was shown that "human

of commercial bovine thrombin.

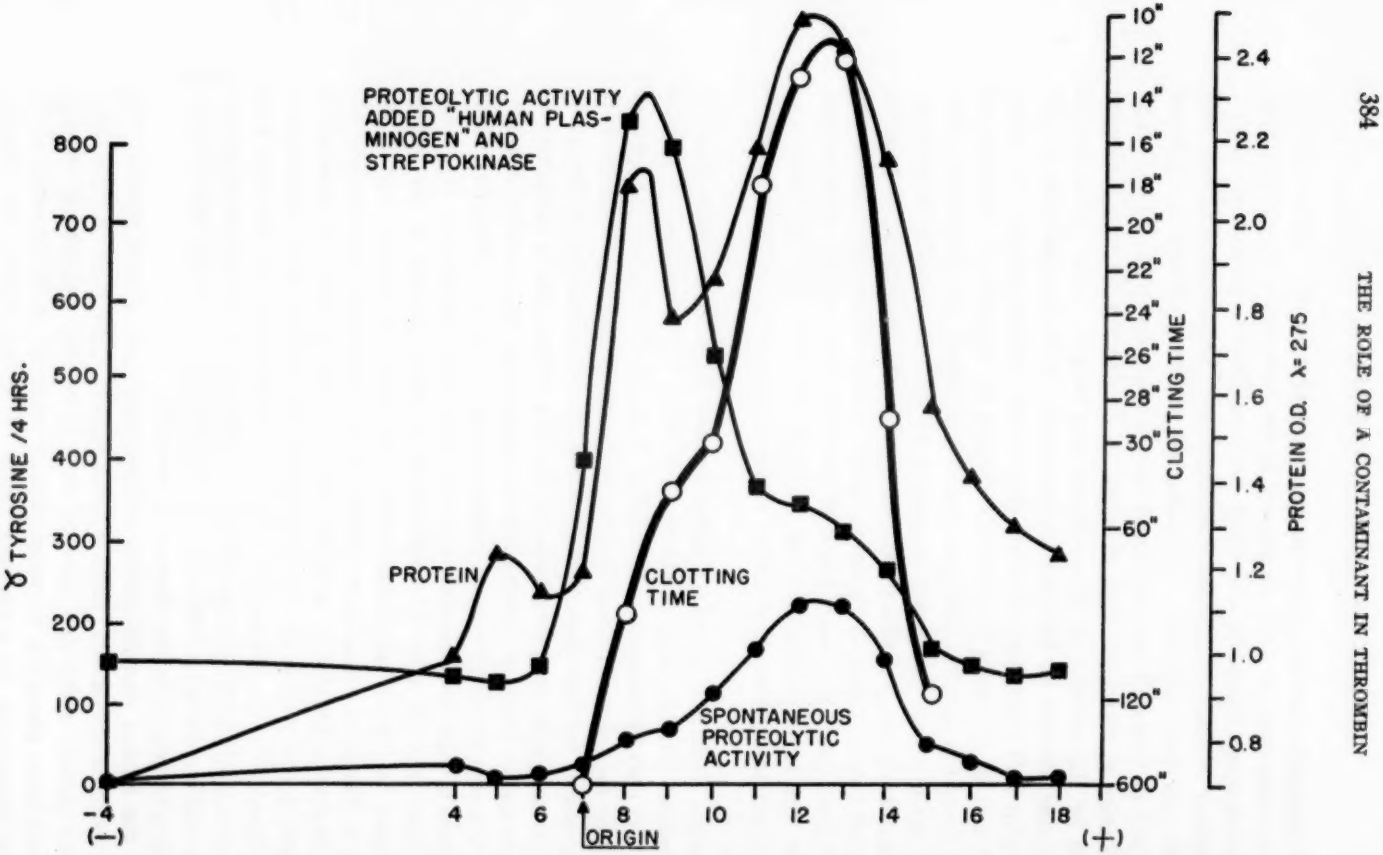

FIG. 2. Properties of starch electrophoresis eluates of commercial bovine thrombin. Proteolytic activities are expressed as in Fig. 1. The clotting time is expressed as the seconds required for 0.1 ml. of eluate to clot 1 ml. of 0.15 per cent bovine fibrinogen in veronal buffer. The scale is inverted so that high activities appear at the top of the figure. Protein concentrations are expressed as the optical density at 275 mμ. 2 ml. of eluate were activated with 0.35 mg. of "human plasminogen" and 150 units of streptokinase.

plasminogen" contains a large excess of proactivator. On conversion to activator it can bring about the activation of plasminogen of human or bovine origin. If the thrombin preparation which is of bovine origin contains a large amount of bovine plasminogen as impurity, then the results of Fig. 1 could be anticipated. To confirm this explanation of the enhanced proteolytic activity of human plasmin in the presence of thrombin, electrophoretic investigation of the thrombin preparation was undertaken. It is to be noted that thrombin itself has some caseinolytic activity, but this is a property of the enzyme itself and is independent of any added streptokinase. At 1.4 units of streptokinase/mg. plasminogen, the proteolytic activity of (thrombin plus plasminogen plus streptokinase) is less than that of the sum of thrombin alone and (plasminogen plus streptokinase) alone. This is probably due to the presence of a small amount of plasmin inhibitor in the thrombin preparation.

Starch Electrophoresis of Thrombin Preparations.—Fig. 2 shows the results of the starch electrophoresis. The protein curve shows the presence of three peaks at 5, 8.5, and 12.5 cm. The thrombin preparation was placed on the block at the 7 cm. mark. The bulk of the protein moved toward the anode. The rapidly moving peak at the 12.5 cm. coincided with the peak in clotting activity. This must be the thrombin peak. At 8.5 cm. a peak was observed in proteolytic enhancing activity. This must be the bovine plasminogen peak. The small peak at 5 cm. of material moving toward the cathode is the small amount of plasmin inhibitor. The low peak at 12.5 cm. of eluate with spontaneous proteolytic activity (or activity in the absence of "human plasminogen" and streptokinase) is due to the caseinolytic activity of pure thrombin, and accounts for the activity observed in Fig. 1 when 0 units of streptokinase was added to the thrombin and plasminogen mixture. 0.1 ml. of the 9 cm. and 0.1 ml. of the 14 cm. eluate were used for clotting a fibrinolytic assay system, each containing the same amount of fibrinogen, "human plasminogen," and streptokinase. These eluates were chosen because each contained nearly the same units of thrombin as demonstrated by clotting time, but the 9 cm. material was rich in proteolytic enhancing factor while the 14 cm. one was practically devoid of it. The tube clotted with the 9 cm. material lysed in 5.5 minutes; that clotted with the 14 cm. material lysed in 10.5 minutes. Therefore, the proteolytic enhancing material is also a fibrinolytic enhancing material—both of which are activities associated with the active enzyme plasmin. All the enzymatic evidence points to the contaminant in thrombin as being identical with bovine plasminogen. A further electrophoretic investigation was undertaken to show their identity.

Electrophoresis of Radioiodinated Enhancing Factor.—Figs. 3 A and 3 B show the results of the starch electrophoresis of bovine plasminogen and of bovine plasminogen to which the radioiodinated plasmin-enhancing factor separated

ogen in veronal buffer. The scale is inverted so that high activities appear at the top of the figure. Protein concentrations are expressed as the optical density at 275 mμ. 2 ml. of eluate were activated with 0.35 mg. of "human plasminogen" and 150 units of streptokinase.

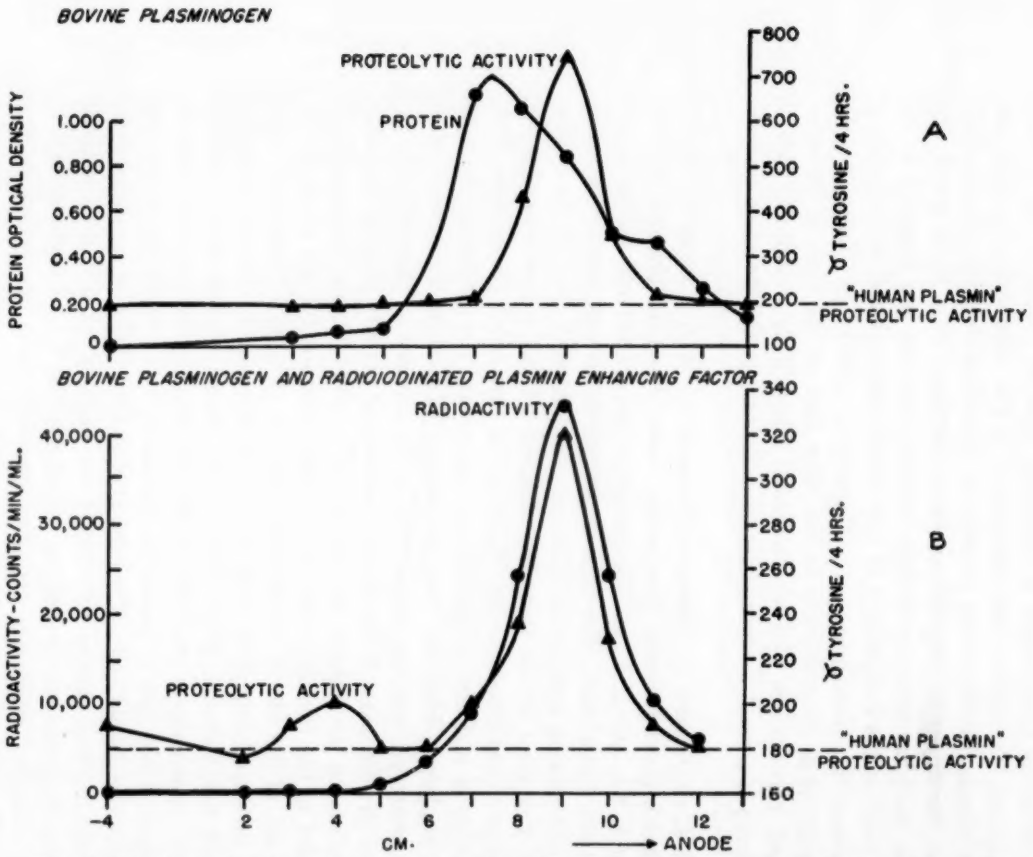

FIG. 3. Starch electrophoresis of A, bovine plasminogen; and B, bovine plasminogen plus radioiodinated plasmin-enhancing factor separated from thrombin. Material was placed on the starch block at 7 cm. and under the influence of the electric field moved toward the anode. 1 ml. eluates of bovine plasminogen were assayed for proteolytic activity after activation with a streptokinase-activated "human plasminogen" preparation. A base line is shown in each figure for the proteolytic activity due to the "human plasmin."

activated "human plasminogen" preparation. A base line is shown in each figure for the proteolytic activity due to the "human plasmin."

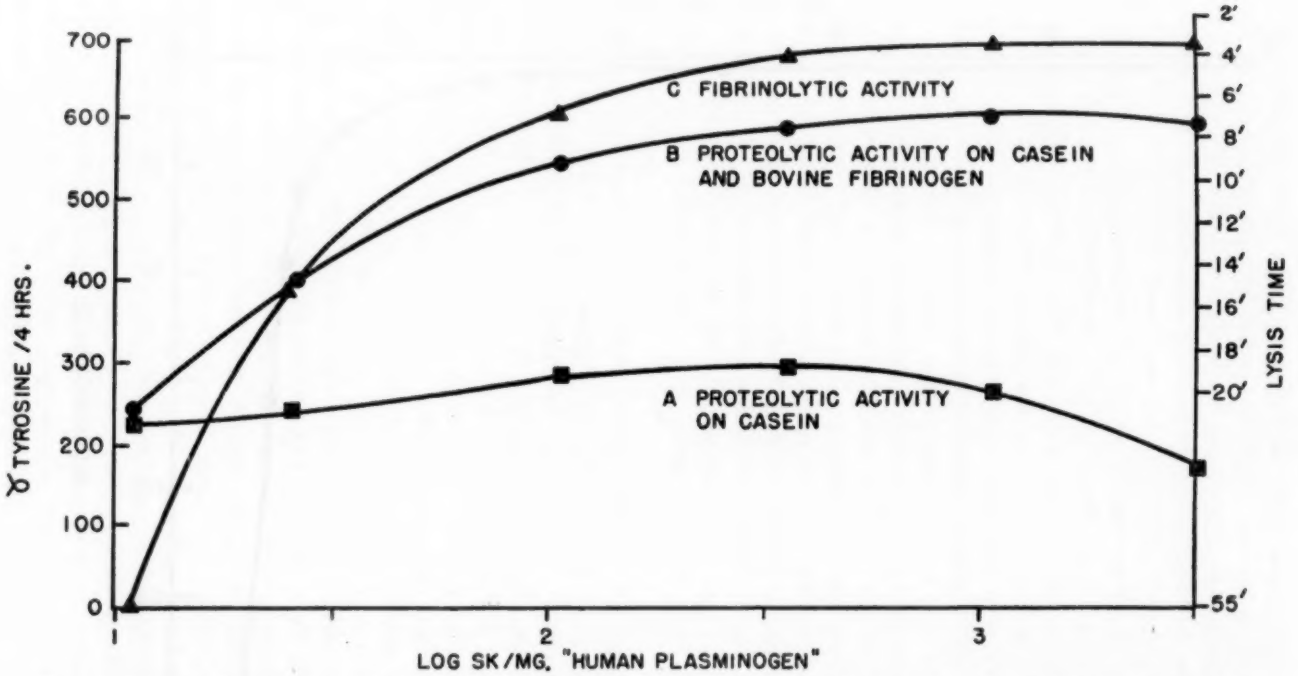

FIG. 4. Increase in enzyme activity of "human plasminogen" with increasing amounts of streptokinase measured on (*A*) casein, (*B*) casein plus bovine fibrinogen, (*C*) bovine fibrin. Proteolytic activities are reported as in the previous figures. Lysis times are reported in minutes. The scale is inverted so that high enzyme activities appear at the top of the figure.

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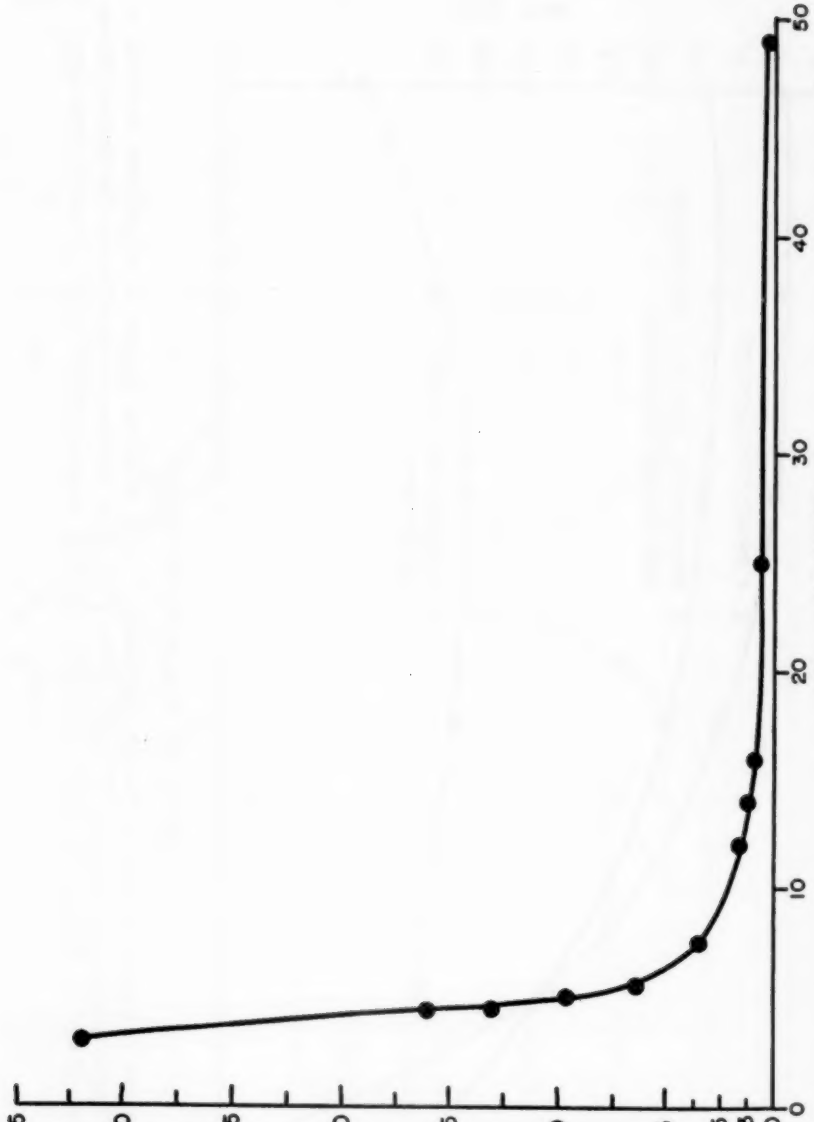

FIG. 5. Lysis time–concentration curve of "human plasminogen" activated with 1000 units of streptokinase.

from thrombin has been added. The bovine plasminogen was not electrophoretically homogeneous, and shows two bumps, as well as a peak, on the protein curve. (Fig. 3 A) The bulk of the protein scarcely moved in the electrophoretic field, but the bumps at 9 and 11 cm. suggest the presence of at least two other components. Since there is a sharp peak in proteolytic activity at 9 cm. the material responsible for the bump here on the protein curve is likely to be the bovine plasminogen. The low specific activity of bovine plasminogen is understandable in view of the large amount of inert protein which makes up most of the bovine plasminogen preparation. In Fig. 3 B, in which 5 mg. of iodinated plasmin–enhancing factor separated from thrombin was mixed with 30 mg. of bovine plasminogen before electrophoresis of the mixture, the proteolytic activity peak again appeared at 9 cm., and coincided exactly with the peak in radioactivity. Therefore, radioiodinated plasmin–enhancing factor from thrombin behaves electrophoretically exactly as does bovine plasminogen. The location of the proteolytic activity peak at 9 cm. in the electrophoretic curves of both the bovine plasminogen alone, and bovine plasminogen plus plasmin–enhancing factor makes it unlikely that the coincidence of the radioactivity and proteolytic activity peaks of Fig. 3 B is due to a complex formation between bovine plasminogen and iodinated plasmin–enhancing factor. Indeed, it is strong evidence that the two substances are identical.

Plasminogen Content of Fibrinolytic Assay Systems.—The experiment reported in Fig. 4 was set up to measure the proteolytic activity of the bovine plasminogen contaminant in a commerical bovine fibrinogen preparation. The same relative amounts of "human plasminogen" and fibrinogen were used in the proteolytic test as were used in the fibrinolytic test. The proteolytic activity of a mixture of "human plasminogen" and streptokinase on casein alone is shown on curve *A*, the proteolytic activity on casein containing bovine fibrinogen on curve *B*, and the fibrinolytic activity (fibrinogen clotted with 50 units of thrombin) on curve *C*. The proteolytic activity in the presence of bovine fibrinogen is only slightly higher than that on casein alone at low streptokinase concentrations and reaches a maximum value which is only twice as great as that of curve *A* at high streptokinase concentrations. This indicated that the proteolytic activity due to the bovine plasminogen contaminant of fibrinogen is just about equal in value to the proteolytic activity of the human plasmin alone. At 36 units of streptokinase the total (human plus bovine) plasmin activity is 400 γ tyrosine/4 hours. At 1000 units of streptokinase, it is 600. Proteolytic activity increased only 50 per cent; yet over that same streptokinase range the lysis time dropped from 15 minutes to 3.5 minutes. According to Fig. 5 this represents a 26-fold increase in plasmin fibrinolytic activity. Bovine fibrinogen appears to contain small amounts of bovine plasminogen in the proteolytic test, yet tremendous amounts are

Fig. 5. Lysis time–concentration curve of human plasminogen

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present in the fibrinolytic test. The fibrinolytic test contains the one additional reagent, thrombin, which is missing from the proteolytic test, and it is this which contains the bulk of the bovine plasminogen. In Fig. 1 the relative amount of thrombin to human plasminogen plus streptokinase is only one-third of the amount in the fibrinolytic test, and yet seven times as much fibrinolytic activity is observed in the presence of thrombin as in its absence at high streptokinase concentrations. At three times this thrombin concentration (the relative amounts in the fibrinolytic test) there would be a 21-fold increase in plasmin proteolytic activity. This is in good agreement with the 26-fold increase observed in fibrinolytic activity when 50 units of thrombin are used for clotting.

Reevaluation of Fibrinolytic Activities.—Interpretation of much of the work in the literature on the components of the fibrinolytic system is based on measurements of fibrinolytic activity. When this activity has been measured on systems containing human plasma fraction and streptokinase the role of the thrombin contaminant can be of prime significance. Until measurements can be repeated on fibrin substrates free of a bovine plasminogen contaminant in thrombin the claims from this laboratory (2) that plasmin is "primarily fibrinolytic rather than proteolytic when compared with trypsin" and that soy bean inhibitor fails to inhibit plasmin fibrinolytic activity must be withdrawn. A few papers (14–16) have seemed puzzling to us, and introduction of bovine plasminogen could account for the results which did not fit in with our general concept of the plasmin system.

Alternate interpretations could be advanced for many other papers appearing in the literature. It is to be hoped that investigators in this field will keep in mind that any experiment in which bovine thrombin has been used for clotting is an experiment which must be closely examined to see whether large amounts of bovine plasminogen fortuitously added could be responsible for the observed results.

SUMMARY

Many of the anomalous results obtained in the fibrinolytic assay of human plasmin systems were shown to be simply explained if bovine plasminogen had been introduced into the assay system on the addition of thrombin. Experimental investigation of the proteolytic and fibrinolytic activity of systems containing plasmin and thrombin showed that enzyme activity was influenced by the presence and quantity of thrombin. The quantity of bovine plasminogen present as a contaminant in bovine fibrinogen was shown to be responsible for only 1/25th of the observed enhanced activity. Thrombin in the amounts commonly used for clotting contained sufficient proenzyme to account for all this activity. A highly purified thrombin preparation obtained from another laboratory, and thrombin purified in this laboratory by starch

electrophoresis brought about no enhancement of activity. The material separated from thrombin by starch electrophoresis was shown to be enzymatically identical with bovine plasminogen and, on labelling with radioactive iodine, was shown to behave physically like bovine plasminogen. Several experiments reported in the literature were reinterpreted in the light of this observation.

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CALCIUM EQUILIBRIUM IN MUSCLE

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(Received for publication, June 22, 1956)

INTRODUCTION

The present investigation was undertaken to determine the permeability of the muscle cell membrane to calcium and to calculate any energy requirement to keep the calcium in a steady state. A preliminary report of this study has already been presented (1).

Methods

General Procedure for Equilibrating Muscles.—The muscles were dissected out of the frogs, and were maintained overnight in Ringer's solution at 5°C. Generally, a set of muscles consisted of the tibialis anticus longus, peroneus, ileofibularis, sartorius, and the semitendinosus. Then one muscle set and its contralateral set were transferred, respectively, to the experimental and the control flasks, each of which contained 100 ml. of solution. Unless otherwise stated, the control solutions were composed of 1.8 mM/liter of $CaCl_2$, 111 mM/liter of NaCl, and 1.34 mM/liter of KCl. After 5 hours with gentle agitation at a temperature of about 23–26°C., the individual muscle sets were blotted on filter paper moistened with Ringer's solution and weighed on a torsion balance. Each muscle set was placed in a platinum crucible, dried at about 100°C. for approximately 1 hour, and ashed overnight at 500°C. The muscle sets usually weighed between 500 and 1000 mg. This technique was essentially the same as described by Reilly (2). The calcium content was determined by either of the following methods:—

Calcium Analysis by Method 1.—The muscle ash was dissolved in 1 N HCl, neutralized with NH_4OH, and then acidified with 0.1 N acetic acid until the color of the solution was orange using methyl red as an indicator. Calcium oxalate was formed with the addition of a saturated solution of ammonium oxalate. Precipitation of calcium oxalate in acetic acid solution has been effective in separating calcium from magnesium (3). The calcium in the calcium oxalate samples was determined by Clark and Collip's modification (4) of the Kramer and Tisdall method (5). In this procedure, the oxalate in the sample was determined by titration with 0.01 N $KMnO_4$. One ml. of $KMnO_4$ solution was equivalent to 0.005 mM calcium.

Calcium Analysis by Method 2.—In this procedure, the calcium in the calcium oxalate was determined by titration with EDTA (ethylenediaminetetraacetic acid) ((ethylenedinitrilo)-tetraacetic acid disodium salt, Eastman Organic Chemicals,

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Distillation Products Industries, Rochester, New York) using the procedure of Flaschka and Holasek (6). The standard procedure was as follows:—

The calcium oxalate precipitate was dissolved in two to five drops of 1 N HCl. One ml. of approximately 0.01 N Mg EDTA was added (or more if necessary). (The stock solution of Mg EDTA was prepared by mixing $MgCl_2 \cdot 6H_2O$ and EDTA together. The pH was adjusted to about 7.0, and the resulting solution was tested to make sure there was no excess Mg or EDTA). Next 2 ml. of buffer (3.55 gm. NH_4Cl plus 22.4 ml. concentrated NH_4OH plus enough H_2O to make a final volume of 100 ml.) was added to bring the pH to about 10, and finally one or two drops of a nearly

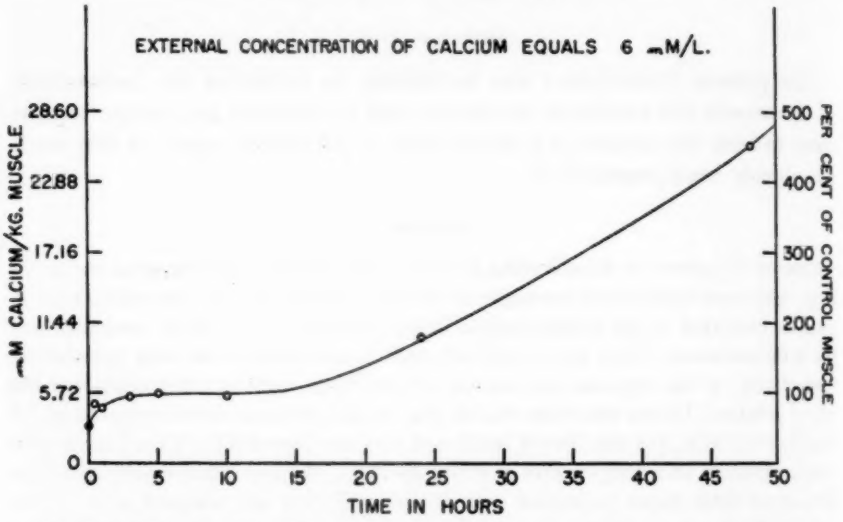

FIG. 1. Effect of equilibration time on the calcium uptake by muscle immersed in Ringer's solution containing 6 mM/liter calcium. Per cent of control muscle was calculated using the average value of 5.72 mM Ca/kg. as equivalent to 100 per cent of the control muscle (see Table I).

saturated, freshly prepared solution of eriochrome black T (1-(1-hydroxy-2-naphthylazo)-5-nitro-2-naphthol-4-sulfonic acid sodium salt (practical), Eastman Organic Chemicals) was added. The samples were titrated with 0.002 M EDTA until the color changed from red to blue. One ml. of the EDTA solution was equivalent to 0.002 mM of calcium. The EDTA solution was made up approximately and standardized daily against a calcium chloride standard. An additional 0.12 μM calcium was added to each sample, since in actual determinations on small and large standard calcium samples this small average amount could not be recovered.

Procedure Using Ca^{45}.—For these experiments the Ringer's solution was composed of 2 mM $CaCl_2$, 111 mM/1 NaCl, 2.68 mM/1 KCl and 2.38 mM/1 $NaHCO_3$. Before use the tissues were kept overnight in Ringer's solution at 5°C., followed by 2.5 to 3.0 hours at room temperature. Thereafter they were soaked for various periods of time in Ringer's solution containing Ca^{45} and were then weighed and ashed in the usual

manner. (The Ca^{45} was obtained from Oak Ridge National Laboratory.) The ash was dissolved in water, transferred to planchets with a diameter of 3.7 cm. and dried. The radioactivity in C.P.M. (counts per minute) was determined using an end-window thin mica (3.5 mg./cm.^{2}) Geiger-Müller tube. The radioactivity for the sample was expressed in per cent of the radioactivity in the Ringer's solution.

RESULTS

The uptake of calcium in muscle immersed in Ringer's solution containing 6 mM/liter of calcium for various periods of time is tabulated in Table I and illustrated in Fig. 1. The control muscles were immersed for 5 hours. The average calcium value for all the 51 controls in this series was 5.72 mM/kg.

TABLE I

Effect of Time on the Uptake of Calcium in Muscle Immersed in Ringer's Solution Containing 6 mM/Liter Calcium

Time		No. of experiments	mM Ca/kg. wt.		Exp./Control	P
Experimental	Control		Experimental	Control		
hrs.	*hrs.*				*per cent*	*per cent*
0	5	7	2.65 ± 0.26	5.09 ± 0.51	53.1 ± 4.2	0.0
0.5	5	6	4.06 ± 0.26	4.97 ± 0.48	83.8 ± 5.5	3.1
1	5	6	4.58 ± 0.18	5.97 ± 0.56	79.3 ± 5.9	1.7
3	5	5	5.14 ± 0.24	5.40 ± 0.20	95.2 ± 3.3	21.8
5	5	6	5.29 ± 0.42	5.51 ± 0.36	97.6 ± 4.5	61.3
10	5	6	5.86 ± 0.69	6.26 ± 0.54	95.8 ± 8.5	64.5
24	5	9	11.3 ± 1.1	6.49 ± 0.47	179 ± 17	0.2
48	5	6	25.1 ± 2.1	5.72 ± 0.40	453 ± 55	0.1
			Average.....	5.72 ± 0.17		

In Fig. 1 this average value was plotted as 100 per cent of the control. After 1 hour, the experimental value was about 80 per cent of the control value. By 3 hours equilibrium was reached and was maintained until 10 hours. When the time increased to 24 and 48 hours, the experimental values increased to about 180 and 450 per cent respectively. At these times definite signs of tissue breakdown were observed and the Ringer's solution became cloudy.

The effect of the calcium concentration in Ringer's solution on the calcium uptake in muscle after an equilibration time of 5 hours is shown in Table II and Fig. 2. The average calcium content of the 71 control muscles (immersed in 1.8 mM Ca/liter) was 2.84 mM/kg. This value was plotted as equivalent to 100 per cent in Fig. 2. When muscles were immersed in a calcium-free solution, the amount of calcium in the muscle was decreased to 1.24 mM/kg. The calcium uptake increased as the external calcium concentration was increased from 0 to 24 mM/liter.

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Eight sets of six sartorii each (800 mg. total weight) immersed in a Ringer's solution containing 2 mM/liter of $CaCl_2$, 111 mM/liter of NaCl, 2.68 mM/liter of KCl, and 2.38 mM/liter of $NaHCO_3$ for 5 hours had a calcium content of 3.32 ± 0.22 mM/kg. A better estimate of the calcium content of sartorii under

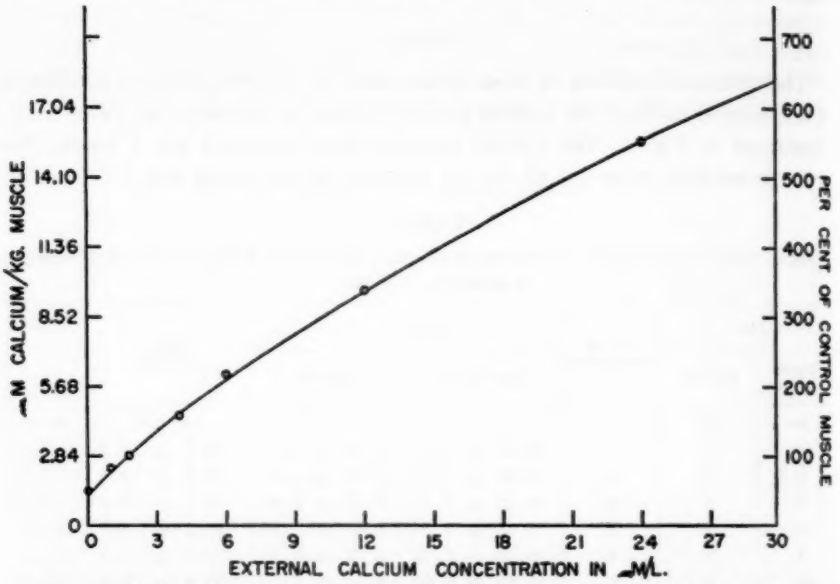


FIG. 2. Relationship between calcium concentration and amount of calcium in muscle after an equilibration period of 5 hours. Per cent of control muscle was calculated using the average value of 2.84 mM Ca/kg. as equivalent to 100 per cent of the control muscle (see Table II).

TABLE II

Effect of Calcium Concentration in Ringer's Solution on the Calcium Content in Muscle

mM/liter Ca in Ringer's		No. of Experiments	mM Ca/kg. wt.		Exp./Control
Experimental	Control		Experimental	Control	
					per cent
0	1.8	12	1.24 ± 0.03	2.53 ± 0.07	49.2 ± 1.7
1	1.8	12	2.41 ± 0.10	2.95 ± 0.13	82.3 ± 3.1
4	1.8	12	3.91 ± 0.18	2.46 ± 0.05	159 ± 6
6	1.8	12	6.03 ± 0.30	2.79 ± 0.11	217 ± 12
12	1.8	12	10.1 ± 0.44	3.04 ± 0.10	337 ± 18
24	1.8	12	17.4 ± 0.62	3.28 ± 0.21	552 ± 39
			Average.....	2.84 ± 0.05	

In all cases, the differences between experimental and control values were significant (P = 0.0 per cent).

these conditions can be obtained from Fig. 2 for which a large number of muscle sets were analyzed. This graphical estimate was about 3.0 mM/kg.

Table III gives the effects of a few conditions on the calcium content. Muscles which were dissected and analyzed without previous immersion in

TABLE III

Effect of Various Conditions on the Calcium Content in Muscle

Condition		mM/liter Ca in Ringer's	No. of Experiments	mM Ca/kg. wt.		Exp./Control
Experimental	Control			Experimental	Control	
						per cent
Freshly dissected	Immersed	1.8	12	1.63 ± 0.07	2.84 ± 0.12	57.8 ± 2.5
Cut	Not cut	1.8	11	5.29 ± 0.10	2.84 ± 0.12	188 ± 5
Cut	" "	6.0	6	11.6 ± 0.4	5.75 ± 0.32	204 ± 8
10 mM/liter EDTA	No EDTA	0.0	11	0.91 ± 0.03	1.12 ± 0.03	81.4 ± 3.1

In all cases the differences between experimental and control values were significant (P = 0.0 per cent).

TABLE IV

The Uptake of Ca^{45} by Frog Sartorius Muscle, Gastrocnemius Muscle, and Achilles Tendon

Time	Sartorius muscle		Gastrocnemius muscle		Achilles tendon	
	No. of experiments	C*	No. of Experiments	C*	No. of experiments	C*
min.						
0.01	8	4.4 ± 0.5	5	2.8 ± 0.4	3	8.6 ± 0.2
0.1	10	8.2 ± 0.6	5	3.2 ± 0.3	3	12.5 ± 3.9
0.5	10	13.7 ± 0.9				
1	10	18.5 ± 1.6	5	5.5 ± 0.5	3	20.9 ± 2.5
2.5	10	36.2 ± 3.6				
5	10	32.6 ± 1.8	5	10.9 ± 2.0	4	37.7 ± 6.1
10	10	44.4 ± 2.5	5	13.1 ± 0.9	3	56.3 ± 5.5
15	10	59.4 ± 3.8	5	15.6 ± 0.6	3	63.4 ± 4.2
30	10	63.5 ± 2.2	5	19.5 ± 3.2	3	66.4 ± 2.0
45	10	68.8 ± 7.9				
60	10	81.9 ± 5.2	5	27.9 ± 3.4	3	87.9 ± 6.3
90	10	68.9 ± 4.8	5	30.9 ± 3.1	3	107 ± 11
120	10	95.4 ± 7.0				
150	10	68.6 ± 3.4				
180	10	73.5 ± 6.0	5	40.9 ± 6.0	3	106 ± 14
300	10	99.1 ± 12.4	4	36.2 ± 3.8	3	102 ± 9
420			2	61.0 ± 10.6	1	130
600	10	93.1 ± 11.7	4	50.8 ± 3.8	3	116 ± 16

$$* C = \frac{\text{C.P.M. per 100 mg. of tissue}}{\text{C.P.M. per 0.1 ml. of Ringer's solution}} 100$$

Ringer's solution contained 1.63 ± 0.07 mM/kg. which was significantly lower than the calcium in muscle immersed in Ringer's solution. Muscles which were cut into small pieces took up about twice as much calcium as their control muscles. A 10 mM/liter solution of EDTA added to calcium-free Ringer's solution removed an additional 0.21 mM Ca/kg.

Table IV gives the uptake of Ca^{45} in sartorius muscle, gastrocnemius muscle, and Achilles tendon when these tissues were immersed in Ringer's solution containing 2 mM/liter. The total uptake in the sartorius muscle appeared to be greater than in the gastrocnemius muscle and less than in tendon.

DISCUSSION

Analysis of Ca^{45} Data.—If a membrane separates two compartments, then the general permeability equation will be:—

$$\frac{dC_2}{dt} = K_1 C_1 - K_2 C_2 \tag{1}$$

(a) Subscripts 1 and 2 refer, respectively, to compartments 1 and 2.
(b) Units of exchange constants K_1 and K_2 are in $time^{-1}$.

In order to integrate Equation 1, it is necessary to know the relationships between time and K_1, K_2, and C_1. The exchange constants may be functions of the chemical concentrations, and if the chemical concentration changes with time, then the exchange constants may also change with time. However, if the chemical concentrations are constant, then it may be assumed that the exchange constants are also constant. This latter situation would, of course, be true during a steady state condition in which there is no net transfer of any chemical substance from one compartment to another. If a trace of a radioactive isotope is introduced into a steady state system, and if C_1 and C_2 represent concentrations of the radioactive isotope, then C_1 and C_2 may vary without affecting either the chemical concentrations in compartments 1 and 2 or the exchange constants K_1 and K_2. For a simple system in which C_1 is constant and $C_2 = 0$ when $t = 0$, the integrated form of Equation 1 is:—

$$C_2 = -\frac{K_1}{K_2} C_1 e^{-K_2 t} + \frac{K_1}{K_2} C_1 \tag{2}$$

In the Ca^{45} experiments, the volume of the Ringer's solution was so much larger than the tissue volume that the radioactive concentration of the Ringer's solution was constant throughout the experiment. The muscles were soaked previously in non-radioactive Ringer's solution long enough to assure a condition close to a steady state (see Fig. 1 and Table I). If the muscle could be considered as a single homogeneous compartment, then the Ca^{45}

uptake should correspond to Equation 2 which involves only a single exponential term. The equation of the radioactive uptake was estimated employing a minimum number of exponential terms, which turned out to be three. The method of calculating this type of equation is similar to that described by Solomon (7). The equation of the uptake by the sartorius muscle is as follows:—

$$C = -38.9\, e^{-0.114\, t} - 41.7\, e^{-0.0107\, t} - 14.4\, e^{-3.86\, t} + 95.0 \tag{3}$$

(a) C is the Ca^{45} concentration of muscle.
(b) t is time in minutes.

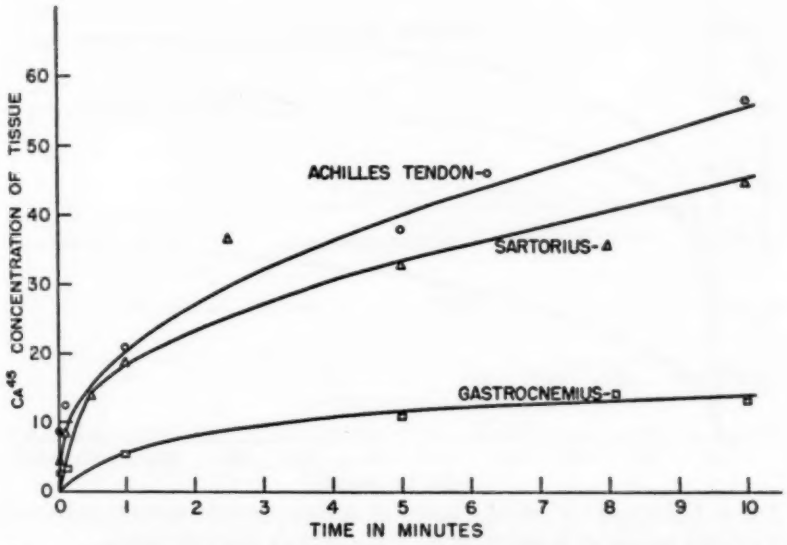


FIG. 3. Calculated Ca^{45} uptake curves of sartorius muscle, gastrocnemius muscle, and Achilles tendon up to 10 minutes. Symbols mark observed points.

The constant 95 was determined graphically. No mathematical analysis is given for the gastrocnemius muscle since diffusion would be slower owing to the thickness of the muscle itself (8) and would more seriously complicate this type of analysis. However, it can be pointed out that the rate of uptake by the gastrocnemius was slower than for the sartorius, probably owing to the diffusion factor. Figs. 3 and 4 illustrate the calculated uptake curve and the experimental points for the sartorius muscle. With so much variability in the data (as illustrated in Figs. 3 and 4) it is evident that Equation 3 represents only a very rough approximation.

Since there were three exponential terms in Equation 3, it was important to determine what type of compartment arrangements could correspond to

such an equation. For a system of N compartments the permeability equation is a single linear differential equation of order N-1 with constant coefficients.

$$\frac{d^{N-1}C}{dt^{N-1}} + a_1 \frac{d^{N-2}C}{dt^{N-2}} + \cdots a_{N-2}\frac{dC}{dt} + a_{N-1}C = a_n \tag{4}$$

(a) C is the concentration in one particular compartment.
(b) Constants a are functions of the exchange constants.
(c) a_n is also a function of the amount in the system.

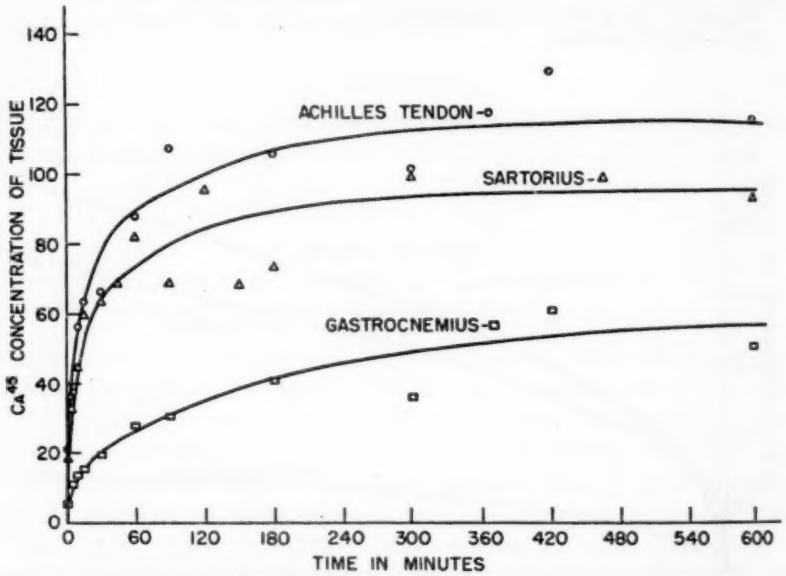


FIG. 4. Calculated Ca^{45} uptake curves of sartorius muscle, gastrocnemius muscle, and Achilles tendon up to 600 minutes. Symbols mark observed points.

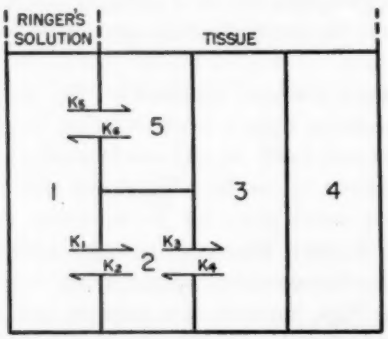


FIG. 5. Diagram showing compartments of tissue. Compartment 1 is the Ringer's solution; compartment 2 is the extracellular space; compartment 3 is the intracellular space; compartment 4 is the non-exchangeable space; and compartment 5 is the surface space.

The integrated form will have a maximum of $(N\text{-}1)$ exponentials and since each exponential contains 2 constants, there will be $2(N\text{-}1)$ constants in the integrated equation. Thus, if the number of exchange constants exceed $2(N\text{-}1)$, then all of the exchange constants cannot be determined unless additional assumptions are made. Since the Ca^{45} uptake curve was described by an equation with three exponentials, a four compartment system containing six exchange constants was chosen as illustrated in Fig. 5. Compartment 1 is the Ringer's solution, compartment 2 is the extracellular space, compartment 3

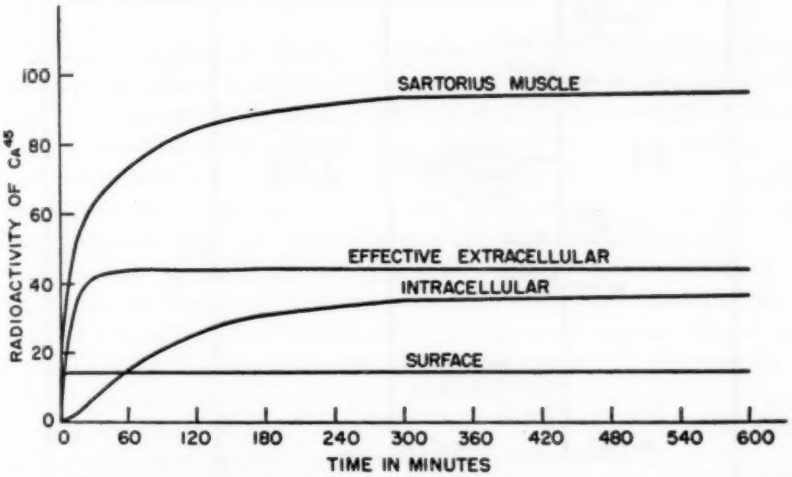


FIG. 6. Calculated Ca^{45} uptake curves of the effective extracellular, intracellular, and surface phases of sartorius muscle.

is the intracellular space, compartment 5 is the surface space, and compartment 4 is the non-exchangeable space. It was found that there were six solutions which would fit these conditions. Each solution contained six exchange constants, but three solutions were disgarded since they contained negative constants. Two other solutions were regarded as improbable because they required the assumption that the extracellular space would become equilibrated before the surface space. The solution finally chosen was the one in which the calcium became equilibrated first with the surface phase and last with the intracellular phase. The kinetics of the uptake of the three phases is illustrated in Fig. 6 and described by the following equations:—

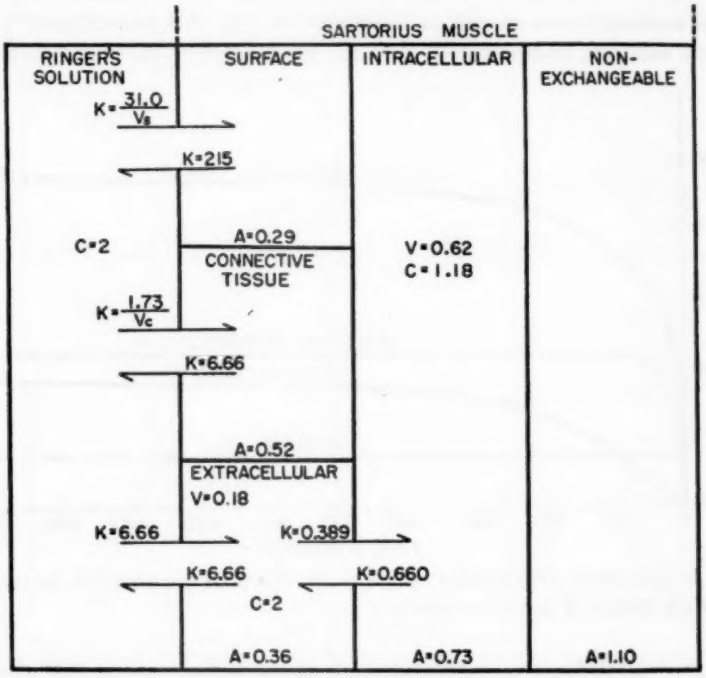
(5) $$C_s = -14.4e^{-3.88t} + 14.4$$

(6) $$C_p = -42.7e^{-0.114t} - 1.3e^{-0.0107t} + 44.0$$

(7) $$C_n = 3.8e^{-0.114t} - 40.4e^{-0.0107t} + 36.6$$

(a) C_s, C_p, and C_n are the Ca^{45} amounts in the compartments 5, 2, and 3, respectively.
(b) t is time in minutes.

The extracellular space as determined by this method is equal to the value of C_p after equilibrium has been obtained. From Equation 6 it appears that the measured extracellular calcium space amounted to 44 per cent. However, this value appeared to be too large and therefore it was assumed that the effective extracellular space actually included some calcium bound in the connective



V IS IN L. H_2O/KG. MUSCLE
C IS IN mM CALCIUM/L.
K IS IN HR^{-1}
A IS IN mM CALCIUM/KG. MUSCLE

FIG. 7. Volumes, concentrations, exchange constants, and amounts of calcium in the different phases of the sartorius muscle.

tissue or not free in solution in the same concentration as in the outside solution. Fig. 7 illustrates the chemical amounts of calcium in the several compartments of the sartorius muscle in the steady state. For these calculations it was assumed that the muscle water was equal to 80 per cent (9) and that the extracellular water space was equal to 18 per cent (10). Any water in the surface and connective tissue phases was arbitrarily included in the extracellular water phase.

If it be assumed that the surface phase is completely equilibrated (and re-

mains so) before any appreciable Ca^{45} enters the extracellular phase and that the extracellular phase is equilibrated (and remains so) before the Ca^{45} enters the intracellular phase, then the mathematics becomes easier and almost identical values are found. Thus, using this approximation, Equations 5, 6, and 7 become:—

$$C_s = -14.4e^{-3.88t} + 14.4 \tag{8}$$

$$C_p = -38.9e^{-0.134t} + 38.9 \tag{9}$$

$$C_m = -41.7e^{-0.0107t} + 41.7 \tag{10}$$

Calcium Localization in Muscle.—It appears that the surface phase became equilibrated in about a minute and contained 0.29 mM calcium/kg. muscle or about 10 per cent of the total muscle calcium. This phase certainly cannot be the extracellular water phase since it equilibrated so rapidly and may be considered perhaps as the epimysium. The extracellular water and connective tissue phases become equilibrated in about 30 minutes, which seems a reasonable period of time. The extracellular water phase was calculated to contain 12 per cent of the total calcium and the connective tissue about 17 per cent. To test the hypothesis that the connective tissue may take up a sizable portion of the Ca^{45}, Achilles tendon was immersed in radioactive calcium solution, and appeared to be able to concentrate the Ca^{45} (Table IV and Fig. 3). Manery (11) has previously pointed out the similarities between tendon and the connective tissue phase in skeletal muscle, and therefore, it seems reasonable to suspect that connective tissue could account for the 17 per cent. It is interesting to note that Harris (12) has reported that the initial loss of Ca^{45} in frog muscle is rapid and exceeds the amount in the extracellular space. Of the total calcium of the muscle 24 per cent was in compartment 3 and exchanged in about 300 minutes. If the assumption is correct that this compartment represents intracellular space then this observation constitutes proof that the cell membrane is permeable to calcium. If it is not intracellular however, then it must be in some slowly exchangeable portion of the extracellular space which seems improbable. The non-exchangeable calcium amounted to 1.1 mM/kg. muscle or about 37 per cent of the muscle calcium. The observation was also made that when muscles were immersed in Ringer's solution containing no calcium, the amount of calcium in the muscle after 5 hours' equilibration varied from 1.12 (Table III) to 1.24 (Table II) mM/kg. muscle. It appears therefore that this non-exchangeable calcium is still in the muscle even after soaking in calcium-free solution. EDTA, a known calcium-chelating agent was added to the Ringer's solution in order to determine whether this non-exchangeable calcium could be removed. However, Table III shows that 0.9 mM/kg. still remained in the muscle even after treatment with 10 mM/liter EDTA. It was calculated from seven experiments of Reilly (2) that 1.78 ±

0.11 mM/kg. of calcium was still present in muscle after immersion in a calcium-free solution.

Energy Expenditure for Maintaining Calcium in the Steady State.—At equilibrium, the total free energy of an ion on both sides of a permeable membrane must be the same. This condition is expressed in Equation 11.

$$RT \ln C_1 + zFV_1 + W_1 = RT \ln C_2 + zFV_2 + W_2 \tag{11}$$

(a) Subscripts 1 and 2 refer, respectively, to compartments 1 and 2.
(b) C is the concentration of the ion (actually the activity should be used instead of the concentration).
(c) R is the gas constant.
(d) T is the absolute temperature.
(e) V is the electrical potential.
(f) z is the valence of the ion.
(g) F is the Faraday constant.
(h) W is the potential due to other forces besides electrical and chemical ones.

The membrane potential may be designated as $V_2 - V_1$ or E_m and the work potential may be designated as $W_2 - W_1$ or W_q. Equation 11 then becomes

$$\ln \frac{C_1}{C_2} = \frac{zF}{RT} E_m + \frac{W_q}{RT} \tag{12}$$

It is finally possible to simplify Equation 12 even further, if some additional definitions are made.

$$\frac{C_1}{C_2} = r^z q \tag{13}$$

(a) $$\ln r = \frac{F}{RT} E_m$$

(b) $$\ln q = \frac{W_q}{RT}$$

If these equilibrium states are used for the steady state in muscle, then the work potential might be considered as an active transport potential, which would be equal and opposite to the passive electrochemical potential. Subscripts 1 and 2 would then refer respectively to the intracellular and extracellular compartments. The value of r equals about 32.9 if the membrane potential is assumed to be 88 mv. (13). If q equals 1, then there exists just a passive Donnan equilibrium; and if q is less than 1, then there is an electrochemical gradient forcing the ion into the cell, which might be counterbalanced by an active transport mechanism forcing the ion out of the cell. If the potassium equilibrium obeys the Donnan equilibrium, then the reciprocal of q for sodium would be identical with Dean's sodium pump factor "e" (14). Using an intracellular concentration of 1.18 mM calcium/liter and an extra-

cellular concentration of 2mM/liter (see Fig. 7), the work factor q was found to equal $5.54 \cdot 10^{-4}$. Being less than 1, this indicates that there is some active force pushing the calcium out of the cell. Actually, the intracellular concentration should only refer to the ionized calcium, and if any is chemically bound, then q would deviate even further from 1. The value for the work potential for calcium was calculated to be -4.37 cal./mM. Landahl (15) calculated that the work function for sodium was $-5.2/RT$ (about -3 cal./mM). Thus the electrochemical gradient pushing the calcium and sodium ions into the cell are roughly about the same. The energy consumed by the active transport mechanism can be obtained by multiplying the work potential by the outward flux. The outward flux for calcium is equal to the intracellular exchange constant times the intracellular concentration or to 0.66 hour^{-1} times 1.18 mM/liter (see Fig. 7). This flux would therefore amount to 0.779 mM/(liter hour), and the active transport energy would amount to 2.1 cal./(kilogram hour) which is only about 1 per cent of the resting energy, assuming that the resting energy output is about 175 cal./(kilogram hour) (16). It should be emphasized that unless the mechanism of transport is known, it is not possible to determine the active transport energy. Thus, in the steady state the energy which is released as the calcium enters the cell may be utilized to force the calcium out of the cell. Then the active transport energy would equal zero. Ussing's exchange diffusion (17) would be an example of this phenomenon. However, if none of the energy which is released by calcium entry, is utilized by the active pump forcing the calcium out, then the active pump would require the calculated 2.1 cal./(kilogram hour). If there is a net transfer of ions, then the minimum active transport energy would equal the net flux times the work potential. It appears from these experiments, however, that the maximum energy required to keep the calcium out of the cell is relatively small no matter what the mechanism is.

From the data given in Table II and Fig. 2, it was possible to determine the effect of the external calcium concentrations on the intracellular calcium concentrations. These data agree roughly with values obtained by Reilly (2). The equation used for the determination of the intracellular concentrations was:—

$$C_i = \frac{A_i}{A_s + A_c + A_i} \cdot \frac{A_T - A_n - EC_e}{I} \tag{14}$$

(a) A_i is the amount of calcium in the intracellular space.
(b) A_s is the amount of calcium in the surface phase.
(c) A_c is the amount of calcium in the connective tissue.
(d) A_T is the total amount of calcium in the muscle.
(e) A_n is the non-exchangeable calcium (1.10mM/kg.).
(f) E is the extracellular water space (0.18 liter/kg.).
(g) C_e is the extracellular calcium concentration.

(h) I is the intracellular space (0.62 liter/kg.).
(i) C_i is the intracellular calcium concentration.

It was assumed that both A_n and the ratio $\frac{A_i}{A_s + A_e + A_i}$ remained constant with any change in C_e. If these assumptions are correct, then by substituting

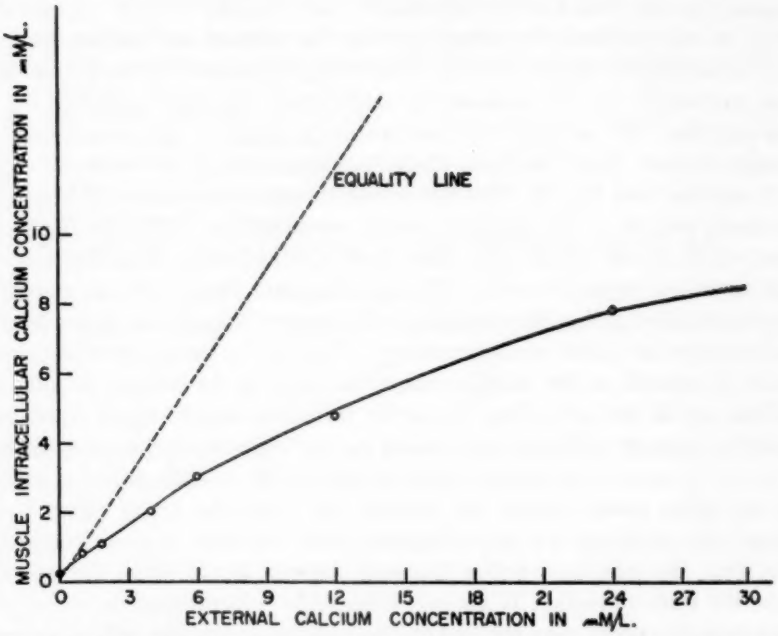


FIG. 8. Relationship between external calcium concentration and muscle intracellular calcium concentration after an equilibration period of 5 hours.

the amounts obtained from Fig. 7 into Equation 14, it becomes possible to calculate C_i.

$$C_i = 0.765\,(A_T - 1.10 - 0.18 \cdot C_e) \tag{15}$$

Fig. 8 shows the relationship between the extracellular and intracellular calcium concentrations. As the external calcium concentration is increased, it appears that the intracellular concentration only slightly increases. Thus as C_e is increased, the ratio of C_i to C_e is decreased. From Equation 13, it can be seen that the product r^2q must also decrease with an increase in C_e. However, the value of r^2 probably increased, since the membrane potential increases with increased external calcium concentration (18). Therefore, the

membrane factor q would definitely decrease as C_e is increased: which would mean that the calcium pump forcing the ion out of the cell is able to do more work as the external calcium concentration is increased.

Further evidence for a calcium pump is given in Table III, in which it is noted that merely cutting the muscle into small pieces increased the calcium content, presumably because of some interference with the activity of the pump. Another factor which would explain part of this observed increase might be calcium-binding by the exposed muscle protein. Weimar (19) has found that frog muscle brei can bind a large amount of calcium, and Weise (20) could not detect any ultrafilterable calcium from rat muscle finely cut up. Fig. 1 and Table I also show that prolonged immersion in Ringer's solution produces a tremendous increase in calcium, with visible signs of tissue breakdown. Again this calcium increase can be interpreted to mean that the calcium pump decreases its efficiency as the cell dies, and that protein breakdown exposes an increased number of binding sites for calcium. Other conditions, in which it is possible to suspect a deterioration of the calcium pump and in which there is observed an increased calcium content are in muscular dystrophy (21, 22), in muscular atrophy produced by denervation, tenotomy, or fasting (23) and in old age (24, 25). Malignant tissues contain low amounts of calcium, but there was observed an inability to exchange calcium (26) which might indicate an impermeability of the membrane.

It should be added that due to the large variability in the data, the various calculations should be taken only as rough approximations. Variation in muscle calcium contents has been previously reported (27). Perhaps experiments in which the loss of radioactive calcium from Ca^{45}-equilibrated muscles is measured, may give better results, since only one muscle would be needed to obtain a complete curve. It would be interesting to observe the effects of changing the external calcium on the exchange constants. Such data might prove very valuable in elucidating the transport mechanism.

SUMMARY

1. A study of the calcium equilibrium in isolated frog muscle has been attempted.

2. When sartorius muscles were immersed in Ca^{45} Ringer's solution, the surface phase took up the Ca^{45} in about 1 minute; the extracellular water space and connective tissue in about 30 minutes; and the intracellular space in about 300 minutes.

3. The percentages of total calcium in the whole muscle immersed in Ringer's solution was as follows: 10 per cent in the surface phase; 12 per cent in the extracellular water space; 17 per cent in the dry connective tissue; 24 per cent in the intracellular space; and 37 per cent as non-exchangeable calcium.

4. The exchange constants of isolated frog sartorius muscle to calcium has been determined. The flux of intracellular calcium in the steady state was approximately 0.8 mM/(liter hr).

5. It appears that there is a calcium pump pushing calcium out of the cell against an electrochemical gradient of about 4 cal./mM of calcium. However, since the flux is low, the maximum energy required per hour to pump calcium out of the cell against this high gradient is only about 2 cal./kg. muscle or about 1 per cent of the resting energy.

Acknowledgment is made to Dr. Tomoaki Asano for his excellent criticisms and for clarifying many of the theoretical aspects of the problem, and to Dr. Aser Rothstein for his general comments.

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REFLEX DEPRESSION IN RHYTHMICALLY ACTIVE MONOSYNAPTIC REFLEX PATHWAYS

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(Received for publication, July 3, 1956)

Fluctuation of excitability in the spinal cord in one sense has been a source of annoyance to most who have employed monosynaptic reflexes for one purpose or another. At an early stage in the exploitation of monosynaptic reflexes it became usual practice to record a number of responses in any given circumstance and to average the lot in the hope of attaining a degree of precision in measurement. In a general way it was recognized that mean monosynaptic reflex amplitude could be different at different repetition rates, but until Jefferson and Schlapp (9) studied repetition for its own sake, most were content to control whatever was at work by maintaining the repetition rate constant and, perhaps, discarding the initial responses of a series. Whilst thinking in terms of single shocks one was dealing in fact with trains of shocks. Feeling that the only logical distinction between single shock and repetitive stimulation could be made at a frequency which is just sufficiently low that one reflex event leaves no measurable impression upon the next, the present experiments were begun with the lowly aim of finding that frequency. The answer was such as unequivocally to show that any attempt to achieve single shock conditions as well as a reasonable degree of precision in the face of fluctuation is quite impractical.

The experiments to be described with very few exceptions were performed upon decapitate cat preparations. The remainder were anesthetized with chloralose-urethane. Monosynaptic reflexes pertaining to a flexor motor nucleus or an extensor motor nucleus were elicited by afferent stimulation of the distally severed nerves to biceps posterior-semitendinosus or the pretibial flexors, and to triceps surae, plantaris, or flexor longus. Recording was done from the appropriate distally severed ventral root, that being either the seventh lumbar or first sacral.

In all but a few experiments recordings were made with the aid of a Grass camera adjusted for moving film. The oscilloscope sweep was not employed. By suitable adjustment of the sweep controls, however, the beam bright cycle was gated to display only the monosynaptic reflex. The resulting records thus had the appearance shown in Fig. 2.

To express monosynaptic reflex amplitude as a function of frequency some

100 successive responses at each desired frequency were measured, and the mean value extracted which then was plotted on the ordinates against frequency on the abscissae. Fig. 1 illustrates a typical experiment, so plotted, in which frequency was varied between 3 per minute (0.05 per second) and 50 per second. Reflex amplitude is expressed in per cent of the mean value at a frequency of 3 per minute. This value was chosen because experiment had failed to disclose any dependent variation in mean amplitude at still lower frequencies. Reflex behavior at frequencies higher than 50 per second (*cf.* reference 1) is considered in a subsequent paper (14).

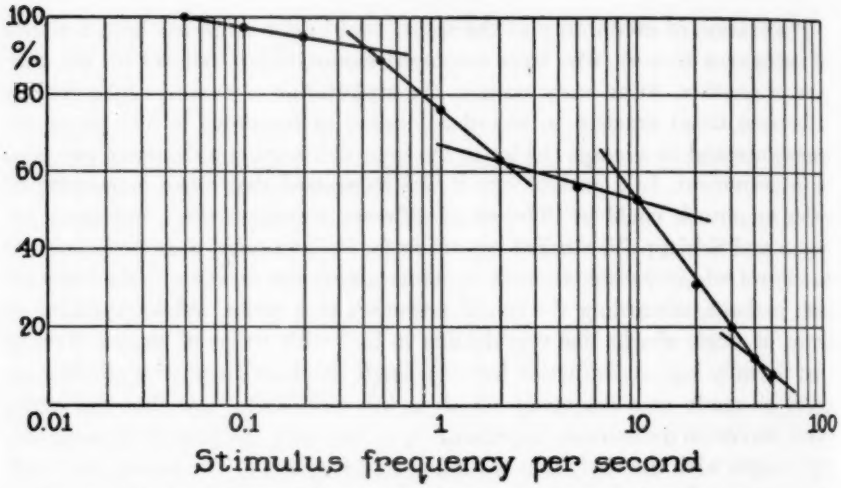

FIG. 1. Relation between afferent stimulation frequency and mean monosynaptic reflex output in the range from 0.05 per second (3 per minute) to 50 per second.

Within the range of frequencies entered in Fig. 1 it is seen that mean monosynaptic reflex amplitude decreased with increasing frequency, itself neither a new nor surprising result. It was not known, however, that the depression of repetition occurred at such low frequencies, since Jefferson and Schlapp (9) had not observed depression with stimulation less frequent than 20 per minute. Now obviously if reflex response is depressed at a certain frequency there must be a period of downward adjustment at the onset of a train of stimuli at that frequency. Because of this fact responses at the onset of a stimulus train either were unrecorded or were discarded in the hope of sampling the steady state condition. Since the significance of the mean monosynaptic reflex amplitude (M) at any given frequency depends upon the establishment of steady state conditions some attention has been directed to finding whether or not this in fact was accomplished.

Establishing the Steady State.—Fig. 2 illustrates the onset of trains of mono-

synaptic reflexes elicited at frequencies varying between 6 per minute (*Q* 10) and 20 per second. By mere inspection it is impossible to say that any period of downward adjustment in amplitude takes place at frequencies lower than 30 per minute (*Q* 2). This is in accord with the observations of Jefferson and

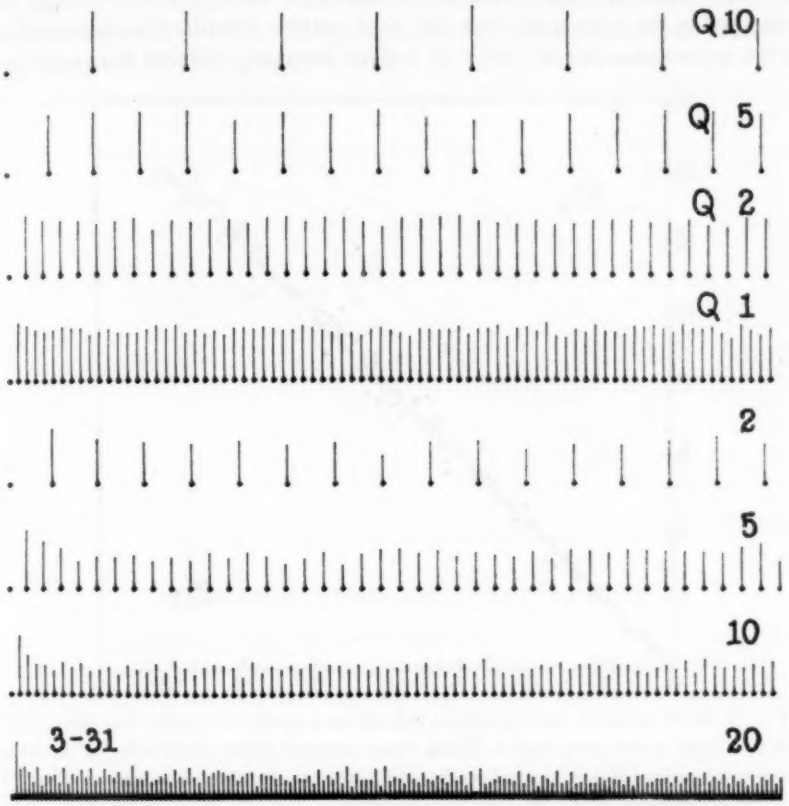

FIG. 2. Onset of trains of monosynaptic reflex volleys at frequencies in the range from 6 per minute (*Q* 10 seconds) to 20 per second to show period of downward adjustment. Film speed for lower four records ten times that for upper four records.

Schlapp (9) who were aware, however, that statistical analysis might reveal depression at lower frequencies of rhythmic stimulation. At higher frequencies than those exemplified in Fig. 2 the initial downward adjustment in amplitude is more dramatic and can be seen, as in the recordings of Eccles and Rall (5), to overshoot the supposed mean steady state value.

Despite the enduring quality of the process underlying depression of response at relatively low frequencies of stimulation the period of adjustment to steady state conditions as far as the eye can tell is rapidly completed. As a

test for the establishment of steady state conditions each series of observations, from which a mean monosynaptic reflex amplitude (M) was extracted, has been divided into two equal component series from which were obtained two mean values M_1 and M_2. Fig. 3 plots M_2 as a function of M_1. The scale of coordinates is arbitrary but consistent in the sense that all values initially were measured in the same units from the photographic records. Absolute amplitude of the mean monosynaptic reflex at a given frequency differed from experiment

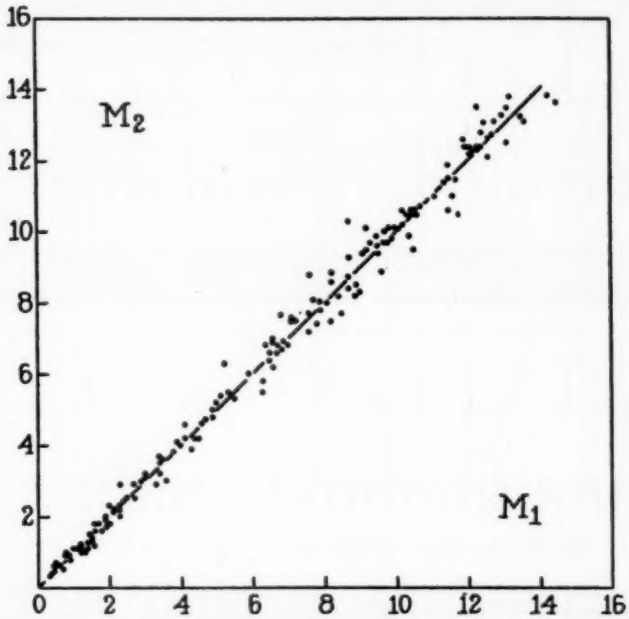

FIG. 3. Each series of monosynaptic reflexes at a given frequency has been divided into two equal component series. Mean monosynaptic reflex amplitude for the second component series (M_2) is plotted on the ordinates as a function of mean amplitude for the first component series (M_1) on the abscissae.

to experiment, as did the amplification employed, but amplification was constant throughout any one experiment. Hence there is no strict relation between the mean values plotted in Fig. 3 and frequency. Nevertheless, in general, the large values represent low frequency responses and the small values high frequency responses. The range of frequencies represented in Fig. 3 is from 3 per minute to 80 per second.

From inspection of Fig. 3 there appears to be no reason to doubt that steady state conditions were established following the initial period of downward adjustment. Two other possibilities existed *a priori*: that iterative monosynaptic reflexes might display a slow increment in amplitude or, alternatively, a slow

decrement. That they did not in the samples is, of course, important from a purely practical point of view. To say that they do not is another matter. It is not unknown for the amplitude of monosynaptic reflexes to drift in one direction or the other during prolonged series of observations. Such changes, however, appear to result from insufficient control of extraneous factors, slight temperature variation being the major known offender, rather than from intrinsic frequency-coupled events.

Relation between Synaptic Drive and Degree of Depression.—The relation be-

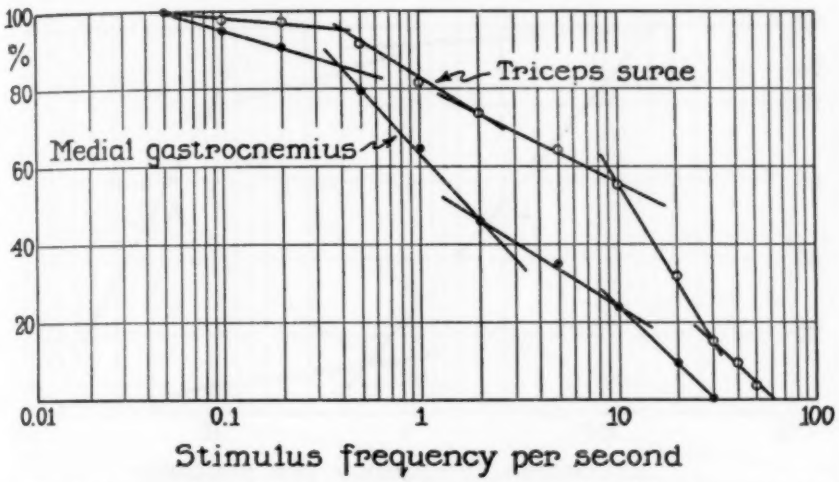

FIG. 4. A comparison of the relation between afferent stimulation frequency and mean monosynaptic reflex output when the entire afferent supply to triceps surae is stimulated and when afferent stimulation is restricted to the medial gastrocnemius component.

tween frequency and amplitude displayed in Fig. 1 is typical. Not unexpectedly, however, degree of depression differs from one experimental situation to another. This is exemplified by the experiment presented in Fig. 4 in which the frequency-amplitude relation was explored initially with afferent volleys in the entire nerve supply to triceps surae and subsequently with afferent volleys restricted to the medial gastrocnemius component. Recording was from the first sacral ventral root that had been tested for content of lateral gastrocnemius motor axons by the use of lateral gastrocnemius afferent volleys. No reflex being present in the resting state, although a small one did appear in the post-tetanically potentiated state, it can be presumed that the reflex elicited by stimulation of the entire nerve pertained essentially, one cannot say exclusively, to medial gastrocnemius. As all other conditions were held constant the important variable was the intensity of synaptic drive upon the moto-

neurons of medial gastrocnemius. In the experimental result depicted in Fig. 4 there is, except for degree of depression, good correspondence between the frequency-amplitude relation of the motor pool at the two intensity levels of synaptic drive.

Magnitude of the monosynaptic reflex elicited from a given motor nucleus, say triceps, by maximal group I afferent volleys may vary considerably from one experiment to the next, a difference that can be accentuated greatly by appropriate manipulation of body temperature. In Fig. 5 are contrasted the frequency-amplitude relations of tricipital monosynaptic reflexes from two

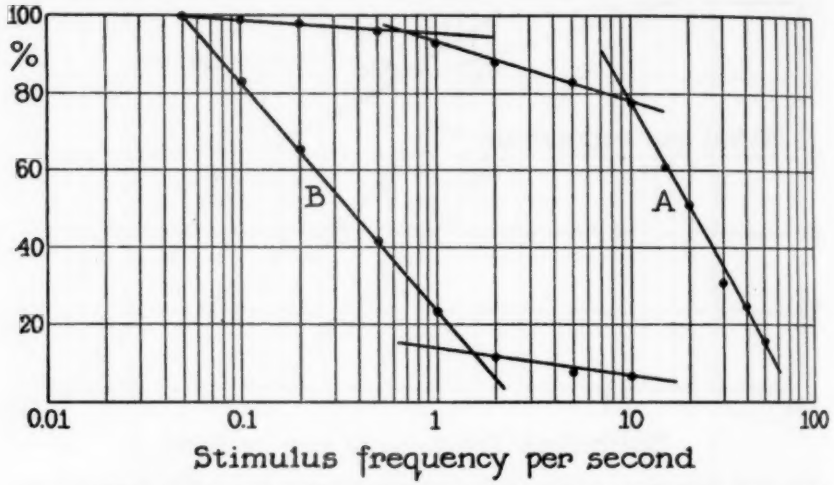

FIG. 5. Relations between afferent stimulation frequency and mean monosynaptic reflex output utilizing (A) a powerful reflex at T = 34°C. and B a feeble reflex at T = 38°C.

preparations one of which, maintained at 34°C., transmitted a powerful reflex, the other of which, maintained at 38°C., transmitted a feeble reflex. The former reflex (curve A) suffers most in the frequency range above 10 per second, although the low frequency depression is distinctly present. By contrast the latter reflex (curve B) comes close to extinction in the range between 1 and 10 per second.

The difference in form between curves 5A and 5B can best be understood with the help of studies upon the behavior, in similar circumstances, of individual motoneurons. To anticipate the findings from study of individual motoneurons (15) it can be said that any monosynaptic reflex includes among its responding member neurons some that are highly resistant to the low frequency depression and others that are highly susceptible. In the first approximation any of the curves in Figs. 1, 4, and 5 can be constructed by varying the ratio between the two sorts of neurons in the discharge zone.

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Two Phases of Depression.—A better idea of the course of depression can be gained by plotting on linear coordinates the frequency-amplitude relations of monosynaptic reflexes. In Fig. 6 this is done. Considering the form of the curves presented it seems a just postulate that one is dealing with two processes, one of relatively short duration that influences the outcome only at frequencies in excess of approximately 10 per second and one of enduring quality. Accordingly it is appropriate to speak of high frequency and low frequency depression

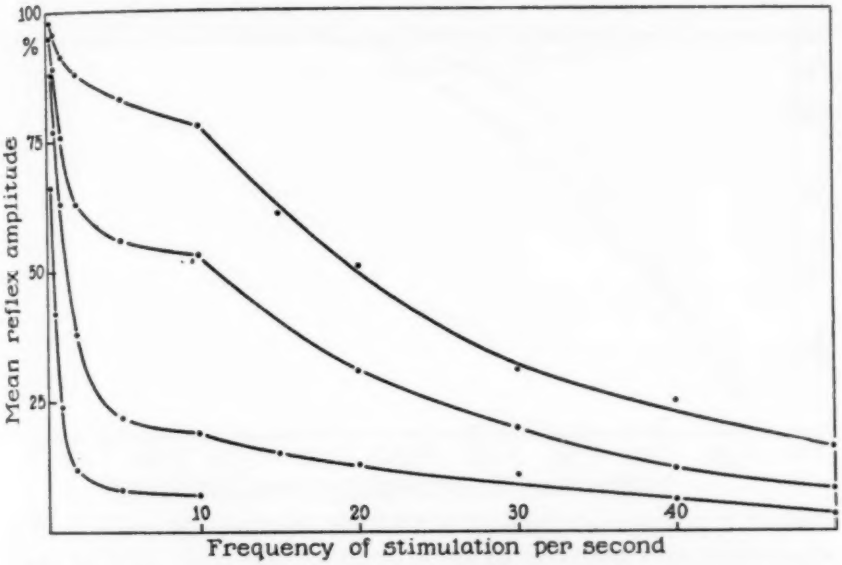

FIG. 6. Linear plots of the relation between afferent stimulation frequency and mean monosynaptic reflex output in four experiments showing differing intensities of depression in the low and high frequency ranges but similarity in the temporal course of depression. Frequency range from 0.2 per second (12 per minute) to 50 per second.

although in assigning the terms it must not be supposed that the now designated low frequency depression does not influence the issue at frequencies higher than 10 per second.

Since the significant discontinuity in the frequency-amplitude curves of Fig. 6 occurs at a frequency of 10 per second one surmises that the high frequency depression is an expression of the influence of the subnormal process in motoneurons.

On High Frequency Depression.—Fig. 7 presents the results of several experiments designed to examine the course of high frequency depression. The method of plotting requires comment. Mean reflex amplitude here is expressed not in terms of frequency but as a function of interval between successive

volleys. This permits a comparison between experiments employing frequency series and experiments employing the conditioning shock-testing shock technique. In order to examine events at the shock intervals of immediate interest and to scale the values from the several experiments amplitude in each at a shock interval of 120 msec. has been designated as 100 per cent and values at other intervals expressed in per cent of that value. The broken line curve charts the course of subnormality as delineated by the conditioning antidromic volley, testing reflex volley technique (12).

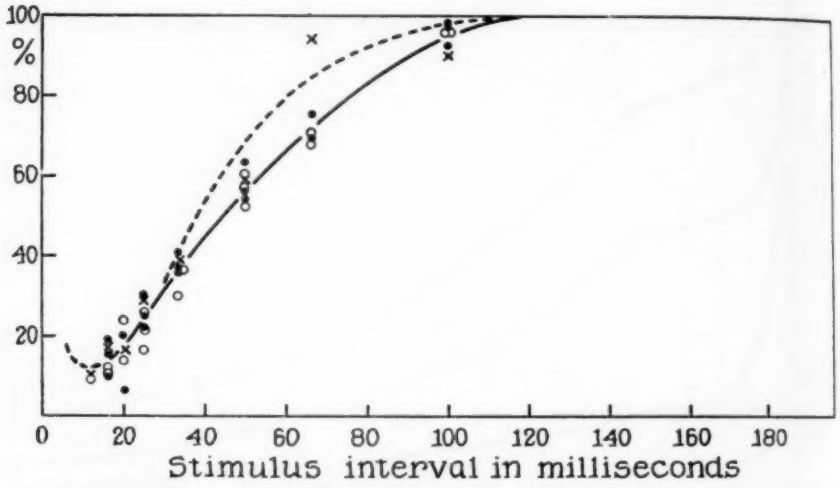

FIG. 7. High frequency depression in flexor and extensor nuclei compared. Mean monosynaptic reflex amplitudes are plotted on the ordinates in per cent of the amplitudes at 120 msec. interval between successive stimuli. The abscissa represents interval between successive stimuli rather than frequency. Results from five experiments are included. Some of the plotted points represent duplicate determinations. The broken line plots course of motoneuron subnormality.

It is clear that the experimental points are very close to the subnormality curve, but it is equally clear that they do not coincide with it. Thus, even if subnormality is the major factor at the shock intervals under discussion, there is yet another factor operating. This, one may suppose is the early part of the process underlying late depression. In view of the experiments of Brooks, Downman, and Eccles (*cf.* reference 4 their Fig. 4 *a*) the point need not be labored except to the extent that comparability of result derived from two-shock analysis and from frequency amplitude series has yet to be established. This question is discussed in connection with Fig. 9.

A more serious question at this juncture is whether the high frequency depression in the steady state is due solely to agencies intrinsic to the monosynaptic pathway, or whether other systems are to some extent contributory. In

fluence by reflex pathways originating in group III fibers can be eliminated from consideration for stimulation did not embrace these higher threshold fibers. However, stimulation necessarily did involve group II fibers and group IB fibers in addition to the monosynaptic afferent group IA fibers. In extensor nuclei the group II fibers, being afferent to a flexor reflex, could contribute to depression but in flexor nuclei would act to mitigate depression. The test, then, for group II afferent fiber contribution is to compare frequency-amplitude relations in flexor and extensor nuclei. This has been done with the result

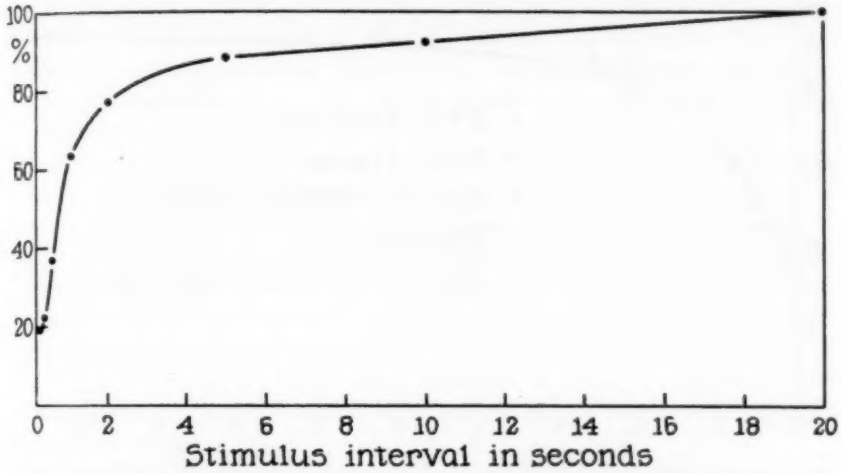

FIG. 8. Time course of low frequency depression obtained by plotting on the ordinates mean monosynaptic reflex amplitude, in per cent of mean amplitude at a frequency of 0.05 per second (3 per minute), as a function of interval between successive stimuli at each frequency studied.

shown in Fig. 7. Open circles represent frequency-amplitude determinations for flexor monosynaptic reflexes, the remaining symbols represent determinations for extensor monosynaptic reflexes. In all five experiments are illustrated.

Since there is no systematic difference between flexor and extensor monosynaptic reflex frequency-amplitude relations in the high frequency depression range one is inclined to discount for that range a significant conditioning role played in the steady state condition by group II flexor reflex connections to motoneurons. It should be noted that Eccles and Rall (5) describe differences during the period of adjustment that could be related to group II action. On the other hand, Jefferson and Schlapp (9) found little distinction between the behavior of flexor and extensor nuclei and even found that addition of sural nerve volleys to gastrocnemius nerve volleys did not change the frequency-amplitude relation of gastrocnemius monosynaptic reflexes.

It is difficult at this stage to rule out participation of the group IB disynaptic

system in the production of the depression of repetition, for in operation this system would be inhibitory to the homonymous motoneurons be they flexor or extensor (10).

Low Frequency Depression.—In order to form a picture of the process that might be responsible for low frequency depression it is useful to plot experimental result in terms of interval between successive stimuli rather than frequency. Fig. 8 presents such a plot and describes, presumably, the time course of the enduring depression.

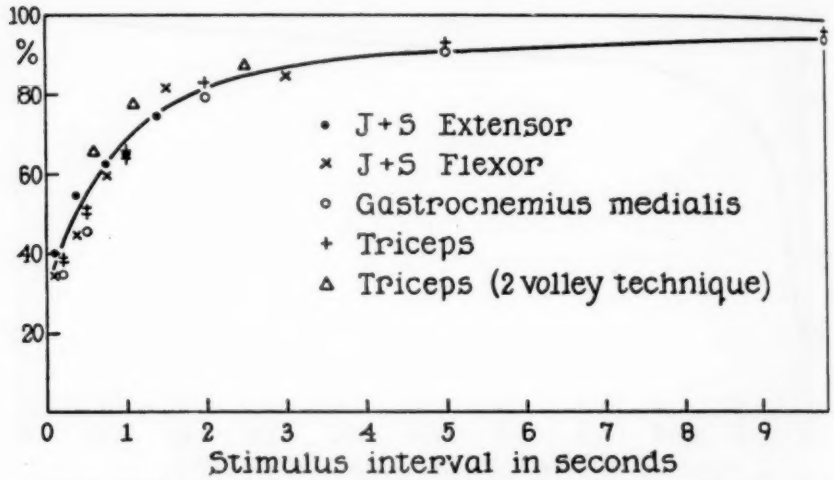

FIG. 9. Comparison of low frequency depression in flexor and extensor nuclei and also of depression as revealed by the two volley technique and by frequency-amplitude series. Method of plotting as in Fig. 8 but note the expanded scale of stimulus interval.

Before discussing further the agency for low frequency depression one may dispose of the question of congruence between the results of two shock exploration and frequency-amplitude series. Again plotted as functions of stimulus intervals rather than frequency in Fig. 9 are the results of three experiments from the present series selected for displaying essentially similar degrees of depression of response. To these have been added the data of Jefferson and Schlapp concerning depression in an extensor nucleus and a flexor nucleus respectively (reference 9, Figs. 1 and 2). Finally there is included the result of an experiment employing the two volley technique, the points representing, on the ordinate, mean amplitude of the second of two maximal reflex volleys for a large number of observations, taken at a repetition rate of 3 per minute to avoid added frequency effects. This is expressed in percentage of the mean response amplitude of regularly interspersed control reflex volleys.

Because the two volley technique reveals a depression identical in time course

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with that evidenced in frequency-amplitude series it seems a valid conclusion that the two techniques do, in fact, yield similar information concerning one and the same fundamental process. Thus one supposes that the low frequency depression described by Jefferson and Schlapp (9) and the so called "subsynaptic depression" of Brooks, Downman, and Eccles (4) represent a single phenomenon, the time course of which is presented in Fig. 8.

That group II and group III afferent fibers in action do not participate as causal agents for the low frequency depression, nor, for that matter, significantly modify the course of depression is sufficiently indicated for the low frequency range by the results of Jefferson and Schlapp (9), which have been confirmed in the present study. By exclusion, then, low frequency depression, just as high frequency depression, can be considered a consequence of action on the part of group I fibers.

Relation between Group I Afferent Input and Degree of Depression.—Granting the role of group I fibers in production of low frequency depression one might hope to eliminate the group IB afferent fibers as significant contributors to that depression. One approach to the problem is to compare the relation between afferent volley size and degree of depression with that between afferent volley size and intensity of some known group IA action upon motoneurons. Suitable bases for comparison are provided in Hunt's (8) study of facilitation and inhibition of monosynaptic reflex discharge in which it was shown that these functions, in approximation, are related in degree linearly with group I afferent volley size.

The results of experiments designed to reveal the relation between afferent input and depression are presented in Fig. 10. Of necessity one must resort to the two shock technique so the validity of the results as concerns low frequency depression necessarily depends upon the comparability of result derived from the use of the two methods. In each experiment the oscilloscope sweep was set at a repetition rate of 6 per minute and all sweeps photographed. The stimulators were arranged so that two successive shocks could be applied to the selected muscle nerve at a fixed interval, that being approximately 500 msec. On alternate sweeps the first, or conditioning, shock was omitted. One beam of the oscilloscope was employed to monitor the conditioning afferent volley, this being recorded at the cord-root junction after conduction centrally from the peripherally located stimulating electrodes. The second beam displayed the monosynaptic reflex responses to the first and second afferent volleys. By this arrangement magnitude of the first afferent volley, which was varied from zero to maximum for the group I band of fibers, gave a measure of input. Magnitude of the monosynaptic reflex response to the measured conditioning afferent volleys gave a measure of output. Finally, comparison between the magnitude of monosynaptic reflex response to the second, fixed, maximal afferent volleys when anteceded by the conditioning volleys, and when not so

anteceded, gave a measure of the degree of late depression imposed by any given magnitude of conditioning volley. The relations between degree of depression and input, obtained in four experiments, are displayed on the left of Fig. 10, the simultaneously obtained relations between monosynaptic reflex output and input on the right.

In the course of experiment it very soon became evident that the relation between group I afferent input and degree of depression was, over a considerable range, linear, but that some secondary effect was influencing the relation

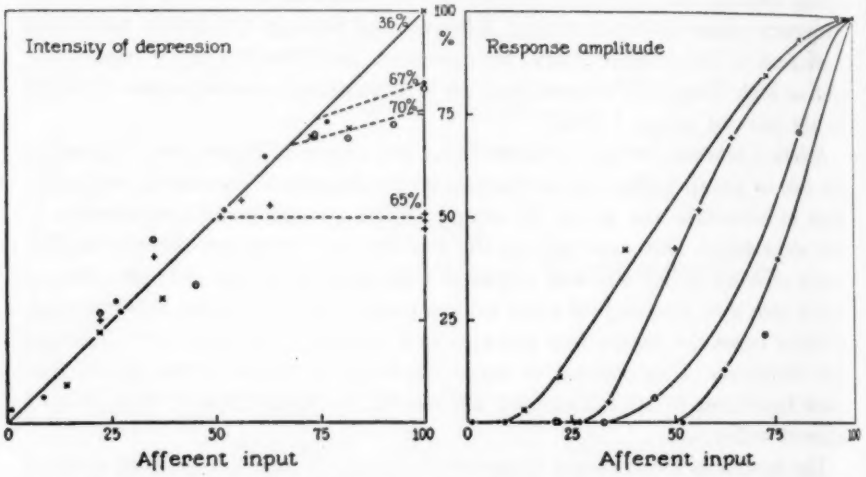

FIG. 10. To the left are plots representing degree of depression of test monosynaptic reflex volleys as a function of measured amplitude of graded afferent group I conditioning volleys timed to antecede the test volleys by a fixed interval. To the right are plots of the monosynaptic reflex output in response to those same measured, graded, group I afferent conditioning volleys. In this way transmission and depression can be compared directly. In all four experiments are represented.

as input approached maximum if the degree of attainable depression was large. In order to present this result graphically straight lines were drawn through the approximately linear portion of the plotted results for each individual experiment and extrapolated to maximal input to give, for each experiment, the greatest degree of depression hypothetically obtainable at that maximal input. The experimental values for degree of depression then were recalculated employing in each case the hypothetical maximum as 100 per cent on the scale of ordinates. This served to scale the linear portions of the input-depression relations from the individual experiments to a common plot, which is given by the solid line on the left of Fig. 10. Such deviations from linearity as occurred in the individual experiments are expressed by the broken line

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continuations from the linear function. At the top of each plot is given in percentage the absolute magnitude of depression realized in each of the several experiments.

It is evident that the smallest afferent volleys exert an enduring depressant action and that this grows *pari passu* with increment in afferent volley size until such volleys are between 60 and 80 per cent maximal. With further increment the degree of depression developed may fall far short of expectation. Some upward convexity of the curve is to be expected, on the basis of Hunt's observations, in the range of group I input above 85 per cent, but the defect in growth of depression with input is, in three of the four experiments, more severe.

In his study of the monosynaptic reflex input-response relation Rall (18) came to the conclusion that the monosynaptic reflex afferent fibers were concentrated in the lower 60 per cent with respect to threshold segment of group I afferent input. Were this so it would provide a convenient explanation for the input-depression curves of Fig. 10 from which one would conclude that depression was strictly limited to origin in action by group IA fibers. Unfortunately for this explanation the simultaneously recorded input-output curves shown to the right of Fig. 10 have the form described by Hunt (8) rather than that described by Rall (18) which is to say that output increases throughout the entire range of input. This implies that the departure from approximate linearity in the input-depression relation cannot be for want of group IA afferent input. It is for this reason that one is led to suppose that some secondary factor in effect places a ceiling upon the realizable degree of depression.

The most likely explanation for the ceiling effect emerges from the fact reported elsewhere (15) that individual motoneurons can be classified, with respect to the influence of low frequency depression, and in the first approximation, into two principal types: those that fall out of the response zone at fairly low frequencies by reason of the low frequency depression and those that are highly resistant to low frequency depression. To account for the occurrence of a ceiling one has only to suppose that the monosynaptic reflexes whose behavior is represented in Fig. 10 pertained to motoneurons some 60 to 70 per cent of which were of the former type, the remainder of the latter type. Thus, as the growing conditioning volleys reached the point of depressing subsequent test response by some 60 to 70 per cent the remaining test response, pertaining to resistant motoneurons, would give little expression to further growth of the conditioning volleys, and the depressive action accountable to them.

The experiments described in connection with Fig. 10 are consistent with the notion that group IA monosynaptic reflex afferent fibers are the agents responsible for low frequency depression, for the relation between input and depression, except for the ceiling imposed, is that to be expected of known group IA action. To the extent that one would expect contribution by group IB fibers,

if anything, to introduce an upward concavity to the curves it may be supposed that group IB fibers are non-contributory in the circumstances of experiment. While this does not constitute rigid proof, significant participation of group IB is unlikely.

One fact concerning low frequency, or enduring, depression is abundantly documented by the experiments of Fig. 10. A comparison of the simultaneously recorded input-depression and input-output relations shows that the depressive action does not involve discharge of motoneurons (*cf.* references 4, 7).

On the Mechanism of Enduring Depression.—An impressive degree of agreement exists concerning location in the presynaptic elements of the agency for low frequency (or enduring) depression in the monosynaptic reflex pathway. The important question then is whether the presynaptic elements act upon themselves so to speak to reduce efficacy, or whether they act upon motoneurons to raise threshold. By use of the designation "subsynaptic depression" Eccles would appear to favor action upon the motoneuron but in attributing it to exhaustion of a chemical mediator (reference 9, discussion) appears to favor reduced efficacy. Evanson (7) suggests as mechanism reduction of the transmitter potentiality of the presynaptic endings, which is to say reduced efficacy.

When in a pathway consisting only of presynaptic and postsynaptic elements one finds a number of phenomena displaying apparently identical intensity-time courses, it is economy of hypothesis to suppose that they may have common origin. In study of the spinal monosynaptic reflex pathway a number of phenomena have emerged that do in fact display apparently identical courses. These are: (1) that member of dorsal root potential sequence designated as D.R.IV.R. (13, 16); (2) the early post-tetanic potentiation that is seen after brief high frequency tetani (6, 13); (3) the recovery of presynaptic structures from anodal type block which characterizes the period prior to onset of late potentiation after more enduring tetani (13); (4) depression in a homonymous pathway after one or several shocks (4, 5, 9, 3, 7); and (5) the low frequency depression of repetitive stimulation. All five of these phenomena on adequate evidence have been assigned to the presynaptic structures. Fig. 11 serves to illustrate the similarity between certain of these phenomena: (1) D.R.IV.R. potential, which is represented by the solid line plotted by tracing on graph paper the original records from two experiments projected at suitable magnification; (2) the depression following a single shock, represented by filled circles; and (3) the low frequency depression observed in two experiments, represented by open circles and crosses.

By its nature D.R.IV.R. represents hyperpolarization of the terminal regions of the presynaptic fibers relative to the polarization level of their more proximal regions. As such it is an appropriate agency for the production of early potentiation following brief tetani, and for anodal type block following more severe tetanization. In giving rise to these phenomena the process underlying

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D.R.IV.R. must be acting in such a way as to alter synaptic efficacy. Thus the intrinsically generated hyperpolarization, in the manner of anodal polarization generally, would augment impulses if not too severe and would when intensified lead to block. Such a reversal of action by intensity change is acceptable according to first principles. It is something else to suppose that when less intense the same process acting in the same way would reverse its action from increasing efficacy, as in early potentiation, to decreasing efficacy, as in enduring depression. This in essence is the observation of Beswick and Evanson (3)

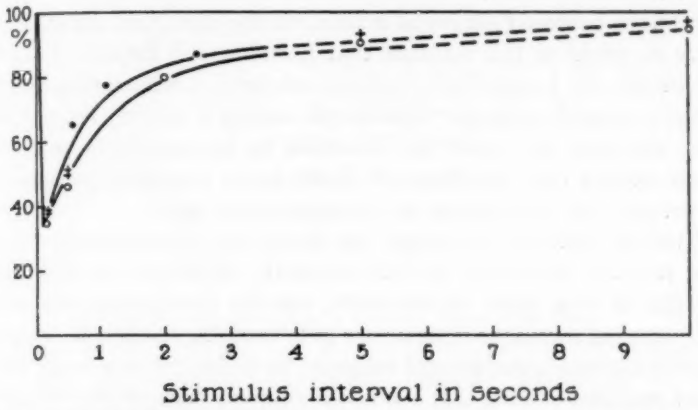

FIG. 11. The D.R.IV.R. potential as recorded from a dorsal root that has carried a short high frequency burst of afferent volleys (*cf.* reference 13, Fig. 10) is represented by the solid lines traced from the recordings in two experiments. Filled circles represent the course of enduring depression according to the two shock technique. Crosses and open circles represent the course of low frequency depression. The broken lines are extrapolations from the recorded course of D.R.IV.R.

who have noted that one volley produces enduring depression of a subsequent test reflex, that two or more volleys intensify the depression, and that further change results in a transition from intensification of depression to its replacement by early potentiation. The alternative then, since influence on the presynaptic fibers themselves leads to potentiation and finally block, is to suppose that the enduring depression results from action of presynaptic fibers not upon themselves but upon the motoneurons.

Such action by the presynaptic fibers upon the motoneurons could be considered as occurring in parallel with the action those fibers exert upon themselves. Following one or a few stimuli action upon the motoneurons would predominate, with hyperpolarization in the terminal regions of the presynaptic fibers becoming more and more significant, as further increase in stimulation takes place, eventually to predominate causing potentiation and finally block.

Such a distinction between two phases of action provides a thinkable basis for the reversal from depression to early potentiation as described by Beswick and Evanson.

If enduring depression is the result of presynaptic action upon the motoneurons one might reasonably expect to find some sign of depression by the use of heterosynaptic reflex testing. Evanson (7) has failed in his search for heterosynaptic depression utilizing the synergic pathways of triceps surae. Similar experiments, forming part of the present series, employing synergic pathways stimulated both at the periphery in muscle nerves and at the dorsal root level have produced equivocal results, for the most part negative.

It may be noted at this juncture that Larrabee and Bronk (11) and also Lorente de Nó and Laporte (17) have encountered in mammalian and turtle sympathetic ganglia prolonged depressions bearing a general resemblance to that here discussed and which are detectable by heterosynaptic test. It is of additional interest that Larrabee and Bronk found in some experiments that the depression was undetected by heterosynaptic test.

Bernhard (2), Brooks, Downman, and Eccles (4), and Eccles and Rall (5) have all reported occurrence of heterosynaptic depression in monosynaptic reflex paths, in some cases inconsistently, but the conditioning test stimulus intervals were not such as to reassure one as to enduring quality. Because of the inconsistent nature of experimental evidence, for which the reasons are obscure, one is not prepared to state that a synergic pathway does exhibit enduring depression. On the other hand, failure to find convincing evidences of enduring depression by means of heterosynaptic test (this being the important reason for proposing, as Evanson has, that depression results from reduced presynaptic efficacy) does not uniquely dispose of the alternative concept that depression results from a depressive action upon the motoneurons. As the matter rests the logical difficulties of the reduced efficacy hypotheses would seem to outweigh the single dubious advantage. This unsatisfactory situation exists, incidentally, whether or not any credence is to be accorded the notion that depression may be linked in some way with other processes that exhibit similar characteristics, notably that underlying the D.R.IV reversal.

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SUMMARY

A study has been made of the depression that occurs when a monosynaptic reflex pathway is subjected to repetitive stimulation.

Reflex depression has a dual origin. High frequency or early depression is postsynaptic in origin and results from subnormality in the motoneurons. Low frequency, late, or enduring depression is presynaptic in origin.

The conditioning volley–test volley technique and the frequency-mean monosynaptic reflex amplitude relation yield similar information concerning reflex depression. Each method has its advantages and for some purposes one or the other of the methods necessarily must be employed.

The results of a variety of experiments are consistent with the proposition that reflex depression in the monosynaptic reflex pathway originates by action in the group IA afferent fibers of muscle origin that are responsible for monosynaptic reflex transmission.

Depression is present at a frequency of 0.1 per second (6 per minute) and absent at a frequency of 0.05 per second (3 per minute). Thus it is impractical for most purposes to employ repetition rates that satisfy the requirement for designation as "single shock" stimulations.

The temporal course of enduring depression has been determined. It is identical with that for a number of other phenomena observable in monosynaptic reflex pathways, which suggests a common origin. The mechanism of low frequency or enduring depression is discussed in the light of this suggestion.

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TEMPORAL SUMMATION IN RHYTHMICALLY ACTIVE MONOSYNAPTIC REFLEX PATHWAYS

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(Received for publication, July 3, 1956)

Problems of spatial summation in short latency monosynaptic reflex transmission have attracted considerable attention in recent years (14, 5, 18, 15, 16, 4). Much of the experimental effort directed toward these problems has been concerned with the relation between input and output and has centered about the two propositions: that the actions at motoneurons of monosynaptic reflex afferent volleys are in degree related solely to the number of active endings involved irrespective of location upon the postsynaptic soma (2, 18, 15, 16) or, alternatively, that location of active endings each relative to the others is an important determinant of synaptic efficacy (14, 5, 4). The most critical evidence now available (4) reaffirms the importance of location and is unequivocally in support of the concept that a brief synaptic action displaying a relatively sharp spatial decrement is responsible for generation of short latency monosynaptic reflex discharge.

Nevertheless, the elements that enter into synapsis, both presynaptic (11, 8) and postsynaptic (2), display enduring electrical change of a sort associable with excitation, and the facilitation of action in the simple pathway is known to have a time constant approximating 4 msec. (6) which is comparable with that of the electrical changes noted. Granting certain assumptions such processes at the synapsis might reasonably be supposed to provide a basis for temporal summation. Indeed the suggestion has been made that "processes similar to Eccles's (1946) 'synaptic potential' or Lloyd's (1946) 'residual facilitation' " (1) could satisfy the requirement for temporal summation in the monosynaptic reflex pathway. However, there exists no evidence to link temporal summation with the process underlying residual facilitation[1] and one is free to make what assumptions one chooses. The experiments to be discussed are presented with the aim of remedying this situation.

[1] Granit (3) has applied the term "residual facilitation" to the increment in action, and response, that follows a bout of high frequency stimulation, namely to posttetanic potentiation (7). Likewise the temporal summation of which he speaks is related to the process underlying potentiation, not to that underlying residual facilitation as the term was originally and is here used. The terminology as here used may not be the best that can be devised, but it does have the merit of giving different names to different things.

Experimental arrangements in general have been those described in the antecedent paper (13), with the important exception that the majority of preparations were narcotized, usually deeply, with nembutal, this for a purpose.

The records of Fig. 1 illustrate monosynaptic reflex responses, or the absence of them, when trains of monosynaptic reflex afferent volleys are engendered, at the indicated frequencies, in the nerves of triceps surae. Recording was from the first sacral ventral root. The preparation was narcotized to the point that

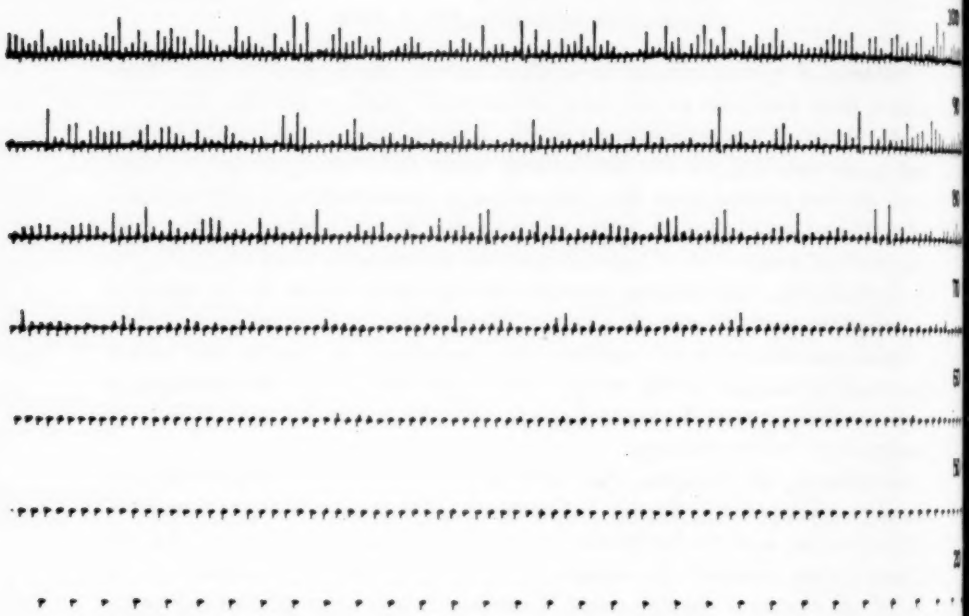

FIG. 1. Monosynaptic reflex behavior of tricipital motoneurons at various frequencies of afferent stimulation, which latter are indicated at the right in stimuli per second. Low frequency response suppressed by nembutal.

single, or low frequency, supramaximal group I afferent volleys did not elicit any monosynaptic, or for that matter polysynaptic, reflex. It is evident from the recordings that some relation exists between frequency and the "amount" of monosynaptic reflex realized at frequencies above 60 per second. The problem is to define that relation.

An initial step in analysis was to determine the mean steady state monosynaptic reflex amplitude at each of the various frequencies of stimulation employed. Fig. 2 contains a plot relating mean monosynaptic reflex amplitude to frequency from an experiment in which response at frequencies from 0.2 per second to 100 per second was examined. For the immediate purpose mean

amplitude has been expressed in per cent of the mean amplitude at a stimulation frequency of 100 per second, which was the maximal value obtained in the circumstances of experiment. As it appears from Fig. 2 there is some justification for supposing that the relation between frequency and amplitude may be approximately exponential within the frequency range under consideration.

With the foregoing in mind the next step in analysis was undertaken with the result depicted in Fig. 3, which contains the results of three similar experiments upon the monosynaptic reflex pathways of triceps surae and upon those

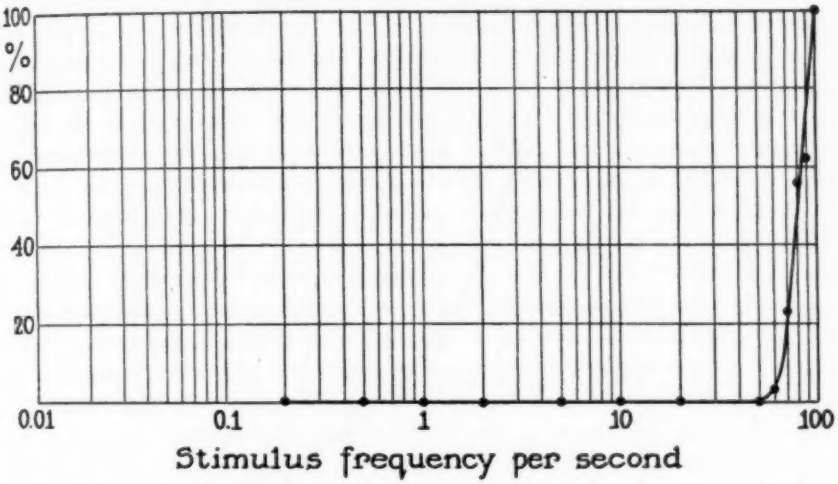

FIG. 2. Relation between frequency and mean monosynaptic reflex amplitude in the steady state. Low frequency response suppressed by nembutal.

of flexor brevis digitorum. The manner in which Fig. 3 is constructed may require some comment. Values for mean monosynaptic reflex amplitude at the several frequencies, and in the several experiments, were plotted on semilogarithmic paper as a function of stimulus interval to find, by extrapolation to zero interval, the factors for scaling the values from individual experiments. The scaled values for monosynaptic reflex amplitude as given in Fig. 3 are represented on the ordinate in per cent of an arbitrary theoretical value for mean amplitude at zero interval, and on the abscissa according to interval between successive stimuli.

The curve plotted in Fig. 3 is the exponential decaying from the assumed arbitrary value at zero time to $1/e$ in 4 msec. It is, therefore, the curve describing in approximation the decay of residual facilitation in monosynaptic reflex paths as originally determined in 1946 (6).

Now it is obvious that a number of secondary influences will come to bear

upon the realizable mean monosynaptic output as frequency increases from the range here employed toward infinity. However, and within the range, it is evident that the decay curve of residual facilitation is an adequate expression for the relation between frequency and mean monosynaptic reflex output *provided* that output is, or approximates, zero at frequencies in the neighbor-

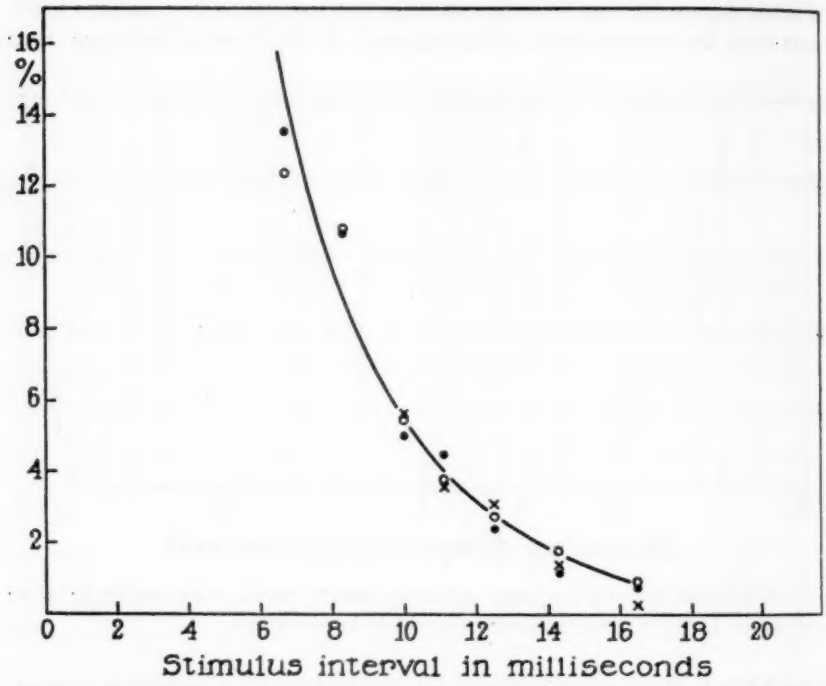

FIG. 3. Relation between frequency and mean monosynaptic reflex amplitude in the steady state plotted as a function of interval between successive stimuli. Results from three experiments scaled by extrapolating plots of log amplitude *vs.* stimulus interval to zero interval. Amplitude is expressed in per cent of an arbitrary value for amplitude at zero interval after scaling. The plotted curve is the exponential decaying from that arbitrary value at zero interval to $1/e$ in 4 msec.

hood of 60 per second, this because residual facilitation decays to zero at conditioning shock–test shock intervals that are the approximate equivalent of that frequency. It will now be clear that the preparations were narcotized to the point of blocking low frequency monosynaptic reflex transmission in order to satisfy the proviso.

According to the present evidence there is sufficient reason for concluding that temporal summation in the rhythmically active monosynaptic reflex pathway is an expression of the same fundamental underlying process as is

residual facilitation, which latter, by the nature of its experimental demonstration (6) has the quality of spatial summation.

The reflex discharges here discussed constitute what Alvord and Fuortes call a "sustained reflex" (1). This being so, the situation raised by the present experiments is rather interesting, for Alvord and Fuortes have contrasted the anesthetized and decerebrate preparations with respect to sustained reflexes thusly: "anaesthetized preparations, though giving reflex reactions to single shocks—can only react with unsustained activity" and "in contrast to what has been found in anaesthetized animals the decerebrate cats examined, regardless of presence or absence of reflexes to single shocks, presented sustained reactions to repetitive stimulation." On the basis of the present experiments it would seem that the two sorts of preparation are not so very different from the standpoint of that which they reveal concerning the workings proper of the monosynaptic reflex pathways. That extraneous influences upon the monosynaptic reflex pathway are very different in the heavily narcotized preparation and the decerebrate preparation is obvious and granted, but such differences can only modify behavior of the anatomical pathway; they cannot be supposed to alter its fundamental intrinsic properties. In view of the differences in extraneous influence it is remarkable indeed that the external expression of monosynaptic reflex behavior is so little different in the two sorts of preparation.

The concept of the sustained reflex has been developed particularly in relation to heteronymous monosynaptic reflex transmission. Heteronymous transmission from one head of triceps to the other, for instance, is something that ordinarily does not occur with isolated or low frequency stimulation but does occur, as Alvord and Fuortes were the first to show, at frequencies above approximately 70 per second (1). Their finding has been amply confirmed during the present series of experiments and extended inasmuch as the high frequency heteronymous response has been found in decapitate and anesthetized preparations although possibly not in the same abundance as in decerebrate preparations.

Until recently it was not known that the heteronymous monosynaptic reflex connections of motoneurons by one means or another could be made to transmit short latency monosynaptic reflexes in response to single shocks. Prior to that discovery one possible, but not unique, interpretation of the facts was that a synaptic property possessed by homonymous connections, by means of which they secured short latency transmission, was lacking in heteronymous connections. Then, upon finding heteronymous transmission by temporal summation during high frequency stimulation, one might be led to suppose that different mechanisms are responsible for low frequency homonymous transmission and high frequency heteronymous transmission and further, by implication, that different mechanisms are responsible for low and high frequency homonymous transmission. This is, if not exactly, at least essentially, the concept of difference between the mechanisms of "unsustained" actions and "sustained" reflexes.

The finding of short latency heteronymous monosynaptic reflex response to single shocks in a variety of circumstances (*cf.* references 10, 12, and contained references) has removed any justification for supposing that other than quantitative differences characterize homonymous and heteronymous synaptic connections. This does not disprove different mechanisms for low and high frequency transmission. It does, however, remove a potential reason for postulating different mechanisms and, further, relieves one of the need for considering homonymous and heteronymous responses separately.

The present experiments substantiate the view that temporal summation in the monosynaptic reflex pathway depends upon operation of the "synaptic potential–residual facilitation mechanism." In doing so they show that temporal summation and sustained reflexes can be accounted for in terms of elementary processes that were discovered initially by the use of single volleys, and which are not peculiar to some particular type of experimental preparation.

Trains of stimuli sometimes have been employed in an attempt to mimic a naturally engendered reflex. Such an attempt can only be self-deceiving for the individual stimuli are synchronous equally with those employed in single shock stimulation, whereas the essence of the natural reflex, with few exceptions, is asynchrony of input. Nevertheless it is fair to ask in what manner asynchrony *per se* could be expected to alter the fundamental properties of synapsis. The essential problem presented by the fact of temporal summation is whether the "residual facilitation process" by summation comes to the point of engendering reflex discharge as is supposed in the concept of sustained reflexes, or whether it becomes merely a more intense facilitator of transmission.

Were the residual facilitation process to be shown capable by summation of engendering discharge in its own right, then, given an asynchronous input, one might argue that the brief synaptic actions documented by Hunt (4) would, by getting out of step, suffer a loss in efficacy that might conceivably parallel the supposed gain in efficacy of the residual process due to temporal summation. Were all this to happen one could then argue that the means of transmission, by reason of quantitative differences, is to some extent different in synchronously and asynchronously repetitive activity.

On the other hand were the residual facilitation process to remain a subliminal threshold lowering agency then, given again an asynchronous input, one might argue that the summation of this process changes threshold to a degree that compensates in some measure for the loss of efficacy suffered by reason of desynchronization of the brief transmitter actions.

By whichever means proposed the reflex product of asynchronous impingement, as in a stretch reflex, would be the same. In fact one cannot by means of present day methods come to any conclusion from observation of asynchronous activity as to the precise nature of the synaptic events responsible for discharge. Thus, while it is legitimate to synthesize the behavior of asynchronous activity making use of synaptic mechanisms demonstrated by the use of syn-

chronous activity, it is perhaps imprudent to deny the existence or importance of some synaptic event from observation of asynchronous activity.

The entire case for the belief that a long lasting synaptic action, now identified with the residual facilitation process, comes by temporal summation to the point of engendering discharge in its own right is built upon evidence obtained by the use of synchronous repetitive volleys rather than asynchronous input, and consists of the observation by Alvord and Fuortes (1) that response in certain circumstances may become randomized in time relative to the incoming afferent volleys. Since the circumstances include the use of extensor motoneurons in decerebrate preparations displaying extensor rigidity, there is ample basis for supposing that events extrinsic to the monosynaptic pathway proper are the essential cause of randomization when this occurs. But this is a problem discussed to greater advantage in connection with the study of response by individual motoneurons for which reason it is deferred until the behavior of individuals has been examined (9).

It must be understood that the present discussion concerning the probability that the residual facilitation process is essentially a threshold lowering agency rather than a transmitting agency need apply only to motoneurons. There is reasonable evidence in ever increasing abundance that some process of a relatively enduring quality, perhaps not unlike that under discussion, is responsible for high frequency repetitive firing in certain internuncial relays (*cf.* for instance reference 17). In this connection it might be well to promulgate the view that motoneurons constitute a rather special class of neurons functionally very important, of course, but numerically an insignificant part of the entire neuron population and not, perhaps, in their patterns of behavior representative of the usual. Surely as much might be said of the monosynaptic reflex pathways. Just as the reflex action served by these pathways is unique so, too, may be some aspects of the mechanism by means of which they perform the service.

SUMMARY

Monosynaptic reflex responses elicited by repetitive stimulation in the frequency range between 60 and 100 per second yield evidence of temporal summation.

The relation between stimulus interval and mean monosynaptic reflex response in the steady state at the frequencies studied is described adequately by an exponential decaying to $1/e$ in 4 msec.

Since the temporal course of spatial summation is described adequately by the same exponential it is concluded that the two phenomena, temporal and spatial, are expressions of the same fundamental process intrinsic to the workings of the monosynaptic reflex pathway, to wit the synaptic potential–residual facilitation mechanism of prior descriptions.

Some discussion of implications is appended.

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MONOSYNAPTIC REFLEX RESPONSE OF INDIVIDUAL MOTONEURONS AS A FUNCTION OF FREQUENCY

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(Received for publication, July 3, 1956)

From time to time questions arise concerning the behavior of motoneurons in some circumstance of reflex activation that cannot be answered by observation of a population of motoneurons. It is then appropaiate to resort to the study of individual members of the population. Even without the pressure of necessity it sometimes happens that the observation of individuals increases one's grasp of the population's workings. To the end that study of individuals will yield an adequate picture of the population behavior one assembles a synthetic motoneuron pool after the fashion of Lloyd and McIntyre (9) and whenever possible devises some test by means of which to compare behavior of the synthetic pool with that of a natural motoneuron pool subjected to similar conditions of excitation. The experiments to be discussed have examined the relation between frequency and monosynaptic reflex response in an assemblage of individual motoneurons constituting a synthetic motoneuron pool.

Reconstituting Pool Behavior.—In a synthetic motoneuron pool constructed of individuals responding to monosynaptic reflex afferent volleys presented at some low repetition rate some individuals respond to every trial and are designated as displaying a firing index (*Fi*) of 100. Others, and these account for some 20 to 30 per cent of the pool (9), display intermediate firing indices. Still others do not respond and hence fall into the $Fi = 0$ category. In the present experiments sequential examination of isolated motoneurons was practiced until an intermediate zone of the same dimensions as that originally found by Lloyd and McIntyre (9) was obtained. By the time this was done a somewhat smaller number of $Fi = 100$ motoneurons had been encountered. The number of $Fi = 0$ motoneurons is not significant for a decision was made not to enter all the $Fi = 0$ motoneurons that could be identified with the aid of post-tetanic potentiation of response, but that did not respond during stimulation at any frequency in the range under study.

For the purpose of assembling the synthetic motoneuron pool a stimulus repetition rate of 30 per minute was used, this being the nearest practical rate to that of the original series, which was 25 per minute. To the left of Fig. 1 is represented, by the solid line plot, the behavior, with respect to firing indices,

of the present synthetic motoneuron pool. For comparison there is plotted by means of the broken line the original synthetic pool of Lloyd and McIntyre adjusted for difference in the number of $Fi = 100$ neurons. The comparison shows the present pool to be relatively a little short in the range from $Fi = 70$

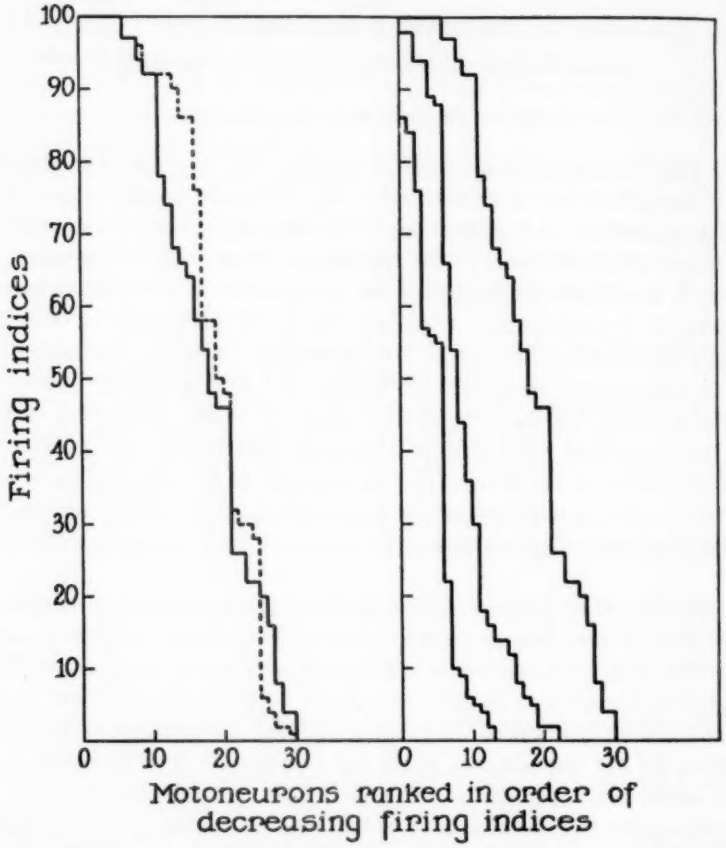

FIG. 1. On the left the solid line plot ranks the individual members of the synthetic motoneuron pool in order of decreasing firing indices. For comparison the broken line represents the original synthetic pool of Lloyd and McIntyre (9) adjusted for difference in number of $Fi = 100$ motoneurons. On the right is represented the response behavior of the synthetic pool in circumstances of varied stimulation frequency.

to $Fi = 90$ and a little high in the range from $Fi = 50$ to $Fi = 70$. Nevertheless all grades are represented and the differences are not great.

From data of the sort contained in Fig. 1 it is possible to calculate in a manner earlier described (9) the average discharge zone which gives the ficti-

tious monosynaptic reflex in the given conditions of activation. To the right of Fig. 1 are three plots that characterize the behavior of the present synthetic pool with respect to firing indices at three frequencies of stimulation. These plots, reading from left to right, represent behavior at 10 per second, 2 per second, and 30 per minute respectively. From the information contained in these plots, and others representing other frequencies, the mean fictitious monosynaptic reflex of the synthetic pool was calculated for a variety of fre-

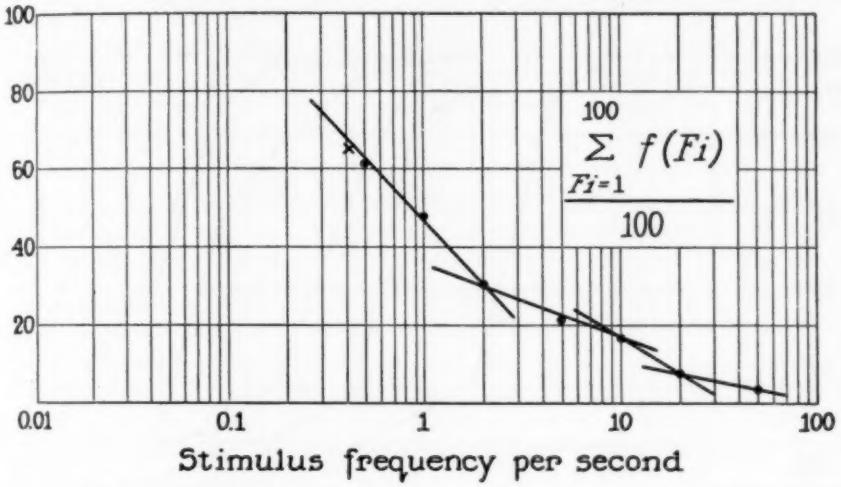

FIG. 2. Average discharge zone (the fictitious monosynaptic reflex) of the synthetic motoneuron pool is here plotted as a function of frequency. *Cf.* reference 11 for natural curves with which this may be compared.

quencies. The result is shown in Fig. 2 which relates amplitude of the mean fictitious monosynaptic reflex:

$$\frac{\sum_{Fi=1}^{100} f(Fi)}{100}$$

to frequency.

Since the relation displayed in Fig. 2 is a close match for the type of relation found when natural pools are the object of study (reference 11, Figs. 1, 4, 5), one may suppose that the method of sequential examination again has proven adequate to secure representative sampling as it has in the study of muscle afferent fibers (3), of motoneurons in the circumstance of post-tetanic potentiation (9), and of motoneurons with respect to the relation of monosynaptic reflex output to afferent input (4).

Some Examples of Individual Behavior.—Fig. 3 presents the frequency-*Fi* relations of five selected individual motoneurons. Before discussing them it is well to bring to mind the fact that postsynaptic subnormality inevitably must limit the firing indices attainable at higher frequencies. Assuming for the moment that the limiting response frequency is 10 per second, based upon an effective subnormal period of 100 msec., then the broken line in Fig. 3 will express the limitation upon firing indices at frequencies between 10 and 100 per second. Actually the limitation is not quite so stringent (*cf.* Fig. 7)

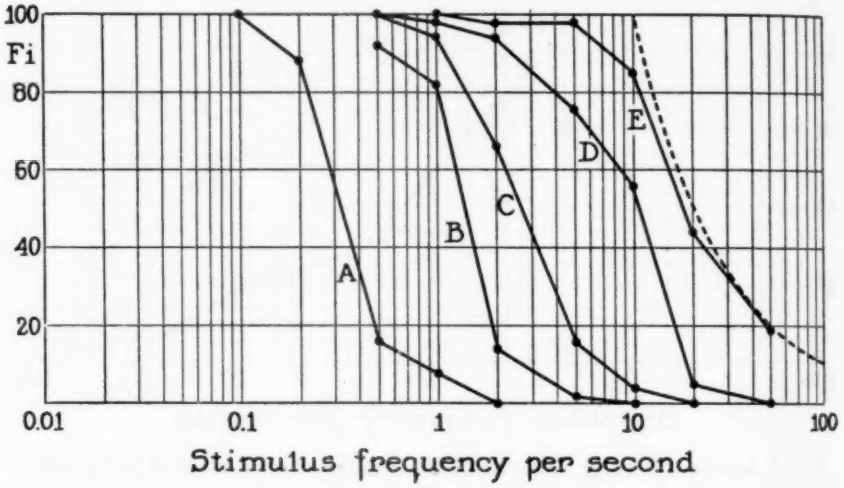

FIG. 3. Frequency–firing index relations of five selected individual motoneurons in the frequency range below 50 per second.

but the value chosen for construction of Fig. 3 is as good as another for the purpose.

The frequency-*Fi* relation for most of the neurons examined lay to the left of that for neuron C of Fig. 3, and of these most did not at any of the frequencies examined approach $Fi = 100$. A number yielded relations resembling that for neuron 3E. The type of frequency-*Fi* relation exhibited by neuron 3D according to present experience is unusual. Thus although the categorization is too rigid it is possible in the first approximation to describe behavior in terms of two typical sorts of neurons: those that behave in a manner rather like neuron 3B and those that behave as neuron 3E. According to previous experience one would say that these neurons, 3B and 3E differ in the transmitter potentiality of their monosynaptic afferent fiber connections (9, 10).

By increasing stimulus frequency neuron 3B, and others of its kind, are brought to $Fi = 0$ at frequencies below that at which postsynaptic subnor-

mality could influence the issue. Otherwise stated transmitter potentiality of the (maximal) afferent volleys is sufficiently low that neuron 3B and kind are removed from the discharge zone by operation of the low frequency (or enduring) depression mechanism. Whilst neuron 3E and kind also suffer from low frequency depression it is quite clear that a major factor limiting response is the high frequency depression due to subnormality.

If now one considers the frequency-amplitude relations for the natural pool monosynaptic reflexes illustrated in Fig. 5 of an antecedent paper (11), it may be realized that the difference between the relations described by curve A and by curve B of that figure may be explained simply by supposing that the two reflexes concerned involved differing proportions of neurons displaying the "neuron 3B type" of behavior and the "neuron 3E type" of behavior. Thus mean reflex amplitude at a stimulation frequency of, say, 10 per second may be considered as giving a rough estimate of the relative number of 3E type motoneurons involved in the particular population monosynaptic reflex response under observation. Furthermore, in experiments utilizing the two shock technique and a variable conditioning volley (*cf.* reference 11, Fig. 10), it is presumably the existence of 3E type motoneurons that places a ceiling on the degree of depression that can result from still incrementing conditioning volleys.

It should be noted that the difference in behavior represented by neuron 3B and neuron 3E is not the result of difference between one preparation and another for both types of frequency-*Fi* curves were readily obtained in one and the same preparation.

Depression and Transmitter Potentiality.—Degree of depression is not a simple function of transmitter potentiality. If the depressing action of an afferent volley impinging upon a motoneuron were simply related to the transmitter potentiality of that volley at that motoneuron then those motoneurons that are powerfully driven should be powerfully depressed and those lightly driven should be only slightly depressed. The end result would be that each individual motoneuron examined would display a frequency–firing index relation much like that of all the others, which manifestly is not the case.

A suitable accounting for the differences in behavior of individual motoneurons is forthcoming if one supposes that degree of depression may be related solely to the number of active presynaptic endings on any given motoneuron just as is facilitation of response in convergent pathways (4). Then, as shown by Hunt (4), with transmitter potentiality related not only to number but to aggregation of active endings the degree of depression could to a considerable extent vary independently of transmitter potentiality. For instance, a motoneuron receiving relatively few monosynaptic connections in a highly aggregated state might yield evidence of its monosynaptic connections possessing a high degree of transmitter potentiality and at the same time

be little depressed in the low frequency range, as is neuron E of Fig. 3. Conversely, a motoneuron fitted with a goodly number of endings, highly dispersed, might have a low firing index and be severely depressed in the low frequency range.

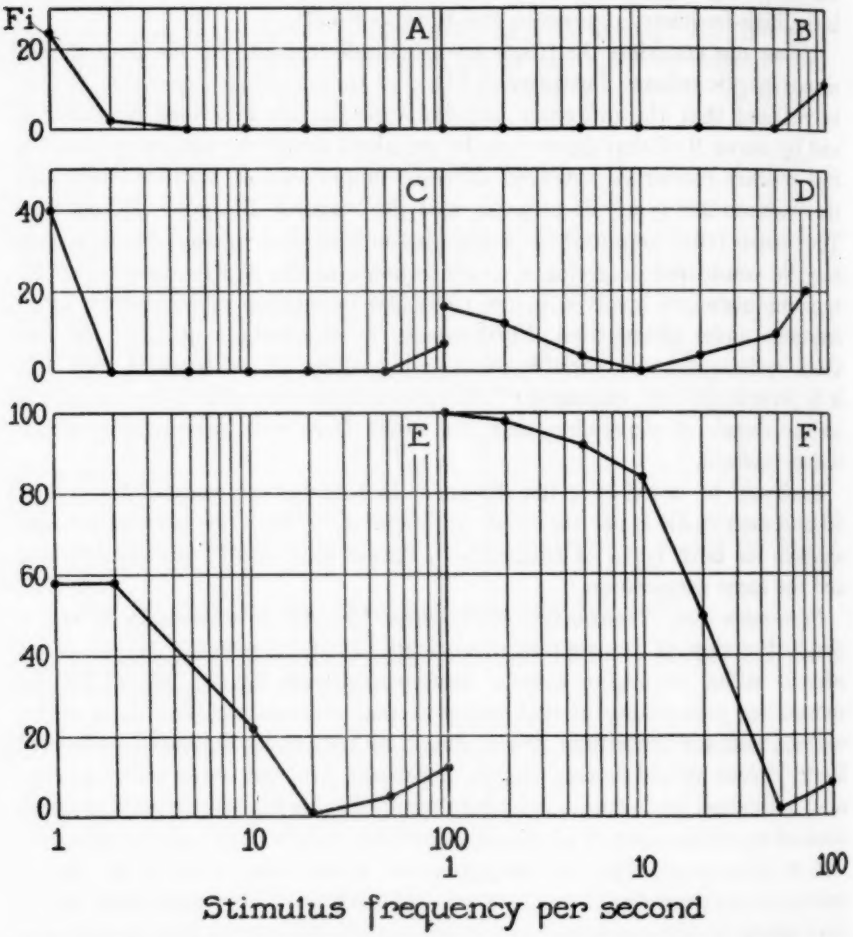

FIG. 4. Frequency–firing index relations of six individual motoneurons in the frequency range between 1 and 100 per second.

Further Examples of Individual Behavior.—Fig. 4 presents instances of individual motoneuron response at frequencies varied between 1 and 100 per second to illustrate certain details concerning temporal summation, high frequency depression (subnormality), and low frequency depression. Neurons 4,

A, B, C, D had been depressed to $Fi = 0$ at a frequency of 10 per second from which it follows that high frequency depression due to postsynaptic subnormality could not have been a factor in these instances of depression. Neuron 4E did have its response incidence curtailed by subnormality in a manner that will be discussed in connection with Fig. 5. For the moment it is necessary only to know that subnormality influenced the issue. Neuron 4F was very obviously and decidedly influenced by subnormality, its frequency-Fi relation, like that of neuron 3E, approaching the subnormality imposed limit.

Subnormality unquestionably placed a ceiling on the high frequency increment in firing index displayed by neuron 4D, for at frequency of 75 per second it was responding on the average every 66.5 msec., but subnormality cannot be invoked to account for any feature of the frequency-Fi relation at lower frequencies. There is no necessity to assume that subnormality placed a ceiling upon high frequency response of the other neurons. Neuron 4A stands in exemplification of the fact that an increment in firing index need not be realized as frequency increases to 100 per second.

If single shock or low frequency response of a natural pool has been suppressed by narcosis (6) or does not occur, as is ordinarily the case in heteronymous pathways (7, 10), one finds that frequencies of approximately 70 per second and higher are requisite for the appearance, by temporal summation, of monosynaptic reflex discharge. In the study of individuals one can, in addition, select for study those that are in the $Fi = 0$ category at low frequencies of stimulation, and by so doing achieve the same condition. Such motoneurons not surprisingly, behave in the same way as does the natural pool that is unresponsive at low frequency. Neuron 4B displays this sort of behavior.

Among neurons that do respond at low frequencies, or to single shocks, one may find that a minimum degree of response is realized at a frequency approximating 10 per second *provided* that subnormality is not a factor in determining response. In exemplification, response incidence of neuron 4D declines progressively as frequency increases from 1 to 10 per second, and increments progressively with further increase in frequency. Neuron 4C is depressed to an extent that the frequency of maximal depression cannot be defined.

An Incrementing Phase of Enduring Depression.—In an antecedent paper (reference 11, Fig. 8) the time course of low frequency, or enduring, depression was plotted for the interval beginning approximately 100 msec. after monosynaptic reflex response and ending with the apparent decay of depression to insignificance at 20 seconds. The course of low frequency depression during the first 100 msec. could not be charted because of interference by subnormality of motoneurons. By the use of properly selected individuals (such as neuron 4D) the impact of subnormality can be avoided and the results obtained show that the low frequency, or enduring depression dis-

plays an incrementing phase to reach maximum at approximately 10 per second or, in terms of stimulus intervals, 100 msec.

In the light of the present findings it is of interest to consider certain responses recorded by Alvord and Fuortes (1) and published in their Fig. 4. On the left side of that figure are simultaneous recordings from the homonymous and heteronymous fractions of triceps at stimulus frequencies of 1, 10, 50, 100, and 150 per second. The heteronymous path requires for discharge a stimulation frequency in excess of 50 per second but presumably less than 100 per second. On the other hand the homonymous path that responds at low (1 per second) frequency and at high (50 per second and higher) frequency displays a minimum approaching extinction of response at 10 per second. Although Alvord and Fuortes were not concerned with the problem of depression their results concur with those here presented in showing that frequencies in excess of 60 per second are requisite for actual temporal summation to occur, but that discharge minimum may be at 10 per second: in short that temporal summation leads into a phase of incrementing depression.

Influence of Subnormality upon the Frequency-Fi Relation.—Each motoneuron that is less influenced than some other motoneuron by low frequency depression is *pari passu* more influenced by high frequency depression. Neurons 4D, 4E, and 4F constitute a series to illustrate the influence upon the course of high frequency response increment of variation in the relative contribution to depression by the low and high frequency processes. Neuron 4D is not influenced by subnormality, neuron 4E is moderately influenced, and the response of neuron 4F is largely determined by subnormality. Each of these three neurons displays a high frequency increment in response incidence, but the frequency at which the minimum response is realized shifts progressively upward as subnormality becomes an increasingly important cause of depression.

Fluctuation and the Frequency-Fi Relation—How Subnormality Can Influence the Issue at Low Fi.—The occurrence of intermediate firing indices depends upon excitability fluctuation in the spinal cord (9). At all frequencies of stimulation above 1 per second neuron 5 (Fig. 5) displayed intermediate firing indices. Furthermore it is obvious from the records that firing index decreased with increasing frequency. At stimulus frequencies 2, 5, and 10 per second subnormality could have no influence on the ability of the neuron to respond. Therefore response is determined by the level of low frequency depression relative to excitability fluctuation.

When subjected to a stimulus frequency of 20 per second the firing index of neuron 5 was a low value due largely to low frequency depression. Nevertheless, within each burst of response the neuron, with one exception, was unable by reason of subnormality to follow the stimulus frequency but could in many instances respond at the first submultiple. At a stimulus frequency

of 50 per second neuron 5 did on a few occasions respond successively at intervals of 80 msec. although its firing index was considerably lower than at a frequency of 20 per second. When frequency of stimulation was increased to 100 per second the neuron was capable on many occasions of responding 60 msec. following prior response, undoubtedly aided by temporal summation. Except for the regular rhythmicity of fluctuation, which is interesting,

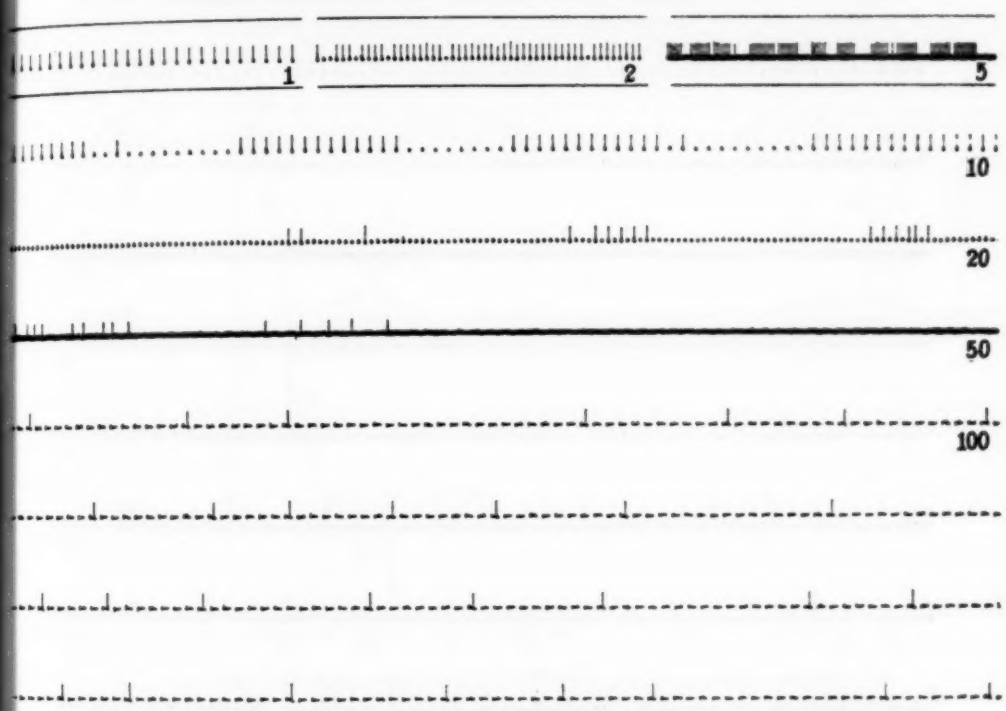

FIG. 5. Responses of a motoneuron at stimulation frequencies varied between 1 and 100 per second. Paper speed increased ten times between 5 and 10 and again between 50 and 100.

unusual, but incidental to the present main point at issue, neuron 5 exemplifies in a simple and usual way the fact that subnormality can influence response at very low firing indices.

Fluctuation and the Origins of Rhythm.—At frequencies below 10 per second rhythm of response depends upon stimulus frequency. So long as the firing index is 100 the rhythm of response is unbroken whether frequency be as low as 1 per second (Fig. 5), or lower, or as high as 10 per second (Fig. 6). The assertion (1) that motoneurons do not fire rhythmically at a stimulus frequency of 10 per second is not confirmed in the present study and indeed

appears to be the consequence of generalization of findings obtained in limited experimental conditions. At whatever stimulus frequency they occur in the range below 10 per second interruptions of rhythmic firing result solely from excitability fluctuation (9).

In the frequency range above 10 per second rhythmic firing may or may not be present and when it occurs may do so in bursts. In this range, however, the rhythm is some submultiple of the stimulation frequency. At the

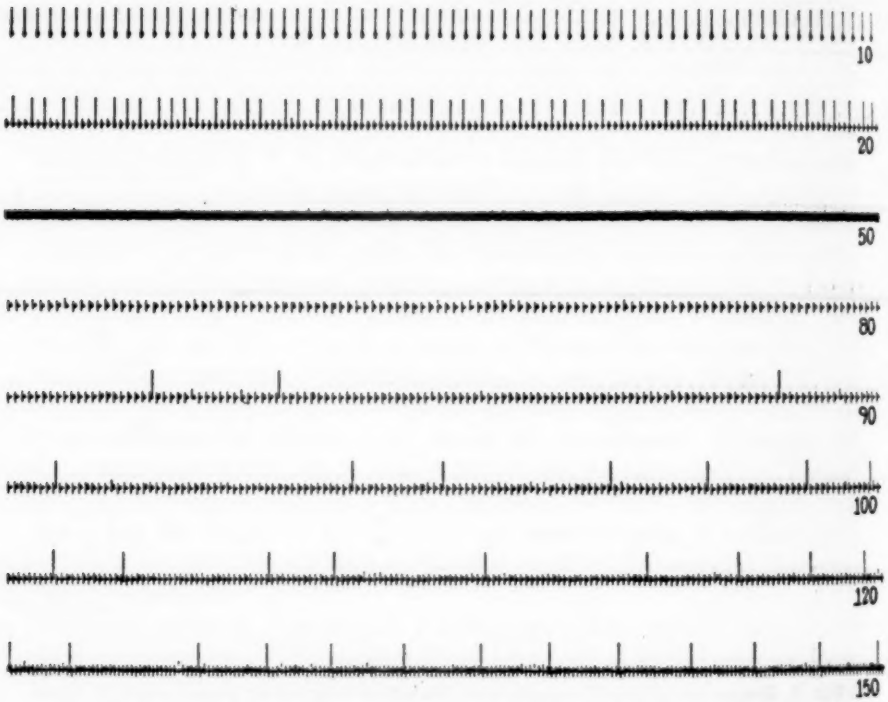

FIG. 6. Responses of another motoneuron at stimulation frequencies varied between 10 and 150 per second. Paper speed increased five times between 50 and 80.

high frequencies of stimulation the steady state level of excitability increases with frequency due to temporal summation (6). As it does so the influence of temporal fluctuation is progressively lessened. In consequence response appears less in the form of bursts and more in the form of regular discharge. Stimulated at a frequency of 100 per second or higher a motoneuron, such as neuron 6 (Fig. 6), may respond just as frequently as it does at 10 per second, but the rhythm is less precise. The reason is simple: a motoneuron receiving volleys each 100 msec. (*i.e.* at a frequency of 10 per second) has no alternative but to respond precisely on the 100 msec. interval, or not to re-

spond, depending upon the instantaneous excitability level in fluctuation. When stimulation frequency is 100 per second the motoneuron is free to respond not only precisely on the 100 msec. interval but also after 90 or 80 msec. as well as after longer intervals depending upon fluctuation. The higher the steady state excitability level established by temporal summation and the less the influence of fluctuation in consequence, the more regular response becomes and the closer response approaches the limit imposed by subnor-

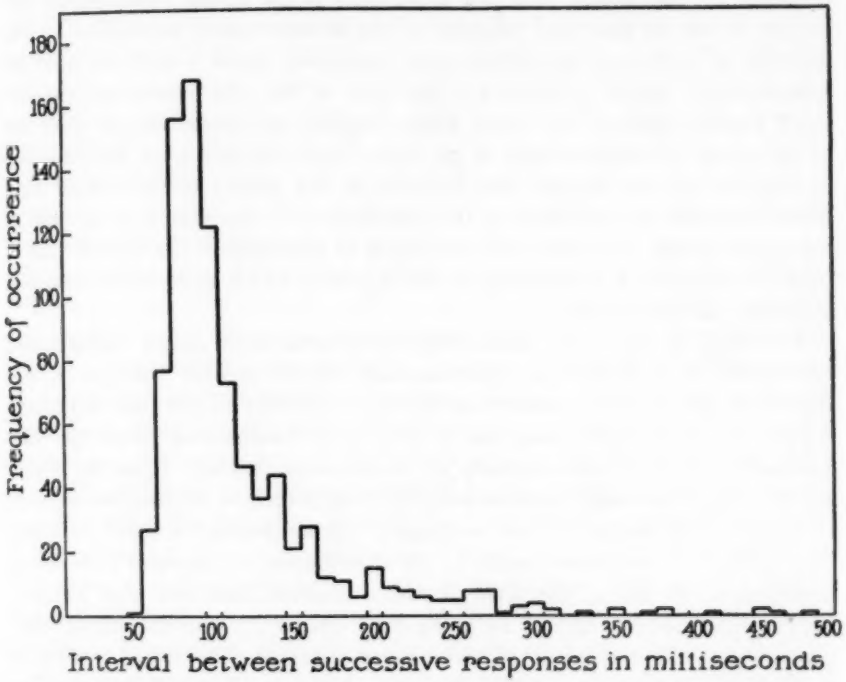

FIG. 7. The frequency of occurrence of intervals between successive responses to a train of monosynaptic reflex afferent volleys at a frequency of 100 per second.

mality. One concludes that the only condition for rhythmic response is that random excitability fluctuation must not influence the issue. Given that condition frequency of rhythmic response is determined by stimulus frequency at low frequencies and at high frequencies by subnormality. Fig. 7 contains a histogram of the frequency distribution of intervals between successive responses at a stimulus frequency of 100 per second. It serves to illustrate graphically the influence of fluctuation upon the ability of neurons to respond rhythmically. In all 915 responses of 10 motoneurons are represented. The distribution is markedly skewed.

Given a certain mean level of excitability and afferent volleys of constant

strength then in total absence of effective influence by fluctuation the motoneurons would respond regularly at intervals of, say, 90 msec. Now, given a slight influence by fluctuation, one can see that a neuron might on some occasions respond after 80 msec. and on some other occasions after 100 msec. depending upon the direction of fluctuation from the mean excitability level. One might expect a motoneuron subjected to slight influence by fluctuation to yield a Gaussian distribution of response intervals. Next, given a greater influence by fluctuation, and this apparently is the actual experimental situation, it will be seen that response of the neuron during fluctuation in the direction of enhanced excitability must inevitably reach a limit imposed by subnormality, which accounts for the form of the distribution toward the short interval side of the modal value. During fluctuation in the direction of decreased excitability there is no upper limit imposed upon the duration of response interval beyond that inherent in the quality of fluctuation itself which accounts for the form of the distribution to the long interval side of the modal value. Otherwise put the degree of skewness in the distribution of response intervals is a reflection of the degree to which motoneuron response is subject to fluctuation.

According to Fig. 7 the modal response interval is 90 msec., which would correspond to a rhythm of approximately 10 per second. Ten per second rhythm is not at all an uncommon finding in activity of the central nervous system. It could result from the occurrence of motoneuron discharge (subnormality) or occur independently of motoneuron discharge (inherent rhythmicity). Fig. 7 provides suitable evidence from which to choose that alternative which is of concern in the workings of the monosynaptic reflex pathway. The absence of prominent peaks in the distribution of response intervals at multiples of 90 msec., the most probable interval, indicates that response intervals are not determined by a rhythmic excitatory process existing independently of the occurrence of motoneuron response. This is not to deny to motoneurons the property of rhythmicity which is well established as a fundamental property of excitable tissue (*cf.* for instance reference 2): it need only mean that the temporally random fluctuation characteristic of spinal cord responsivity (9, 12) is of a magnitude such as to dominate behavior. This also is not to deny that 10 per second rhythm, or indeed rhythm at other frequencies can in other situations be the consequence of inherent rhythmicity.

On Shock Response Interval.—At no time has response of a motoneuron subjected to high frequency afferent bombardment occurred either out of relation to the afferent volleys or with a latency that is not appropriate for short latency monosynaptic reflex discharge. This is in accord with some of the findings of Alvord and Fuortes (1), but not with others. The essential conditions under which Alvord and Fuortes observed a lack of strict relation

between shock and response were stimulation with weak shocks, or of heteronymous afferent fibers, in decerebrate preparations. Since they considered only results obtained from preparations that displayed extensor rigidity it may be assumed that the motoneurons studied (gastrocnemius) were subjected to a heavy excitatory bombardment from supraspinal centers.

The question now is whether random discharge to high frequency iterated stimulation, when it does occur, does so as the result of action intrinsic to the monosynaptic reflex pathway as is, in essence, the proposal of Alvord and Fuortes, or whether impingement from sources extrinsic to the monosynaptic reflex pathway controls the timing of discharge. The mere fact that monosynaptic response of motoneurons at high stimulation frequencies has not become random with respect to afferent volleys in spinal and anesthetized preparations and has in the rigid decerebrate preparations of Alvord and Fuortes is presumptive evidence that the random and extrinsic bombardment that is essential for the development of decerebrate rigidity is likewise the essential ingredient leading to random discharge.

In agreement with Alvord and Fuortes one may believe that it is difficult to bring random bombardment such as that of decerebrate rigidity under experimental control. However, it is possible to introduce a controlled asynchronous bombardment from a source extrinsic to the monosynaptic pathway itself and to combine this with monosynaptic reflex volleys possessing any chosen degree of transmitter potentiality for a given motoneuron under observation. A means for doing this is to stimulate the brachial plexus which in action provides to the motor nucleus of gastrocnemius, for instance, a dispersed internuncial excitatory barrage that waxes and wanes over a considerable interval of time and which may of itself, at the peak of intensity, secure some motoneuron discharge (5, 8). Monosynaptic reflex afferent volleys then are delivered so as to arrive after the onset of internuncial bombardment and before the onset of long spinal reflex discharge, if such indeed does occur.

When all this is done (10), it is found occasionally that the combination of heteronymous monosynaptic reflex afferent volleys and long spinal reflex barrage, each severally ineffective, may lead to instability of interval between monosynaptic reflex afferent volley and response. Prior tetanization of the monosynaptic reflex afferent pathway leads to increased incidence of response and to fixation of response in time relative to the monosynaptic reflex afferent volleys. All responses to homonymous and heteronymous afferent volleys potentiated by prior tetanization, or not, as the case may be, but secured in the absence of long spinal reflex barrage, are linked in appropriate short latency reflex timing with respect to afferent volleys. In other words the presence of extrinsic dispersed bombardment appears in the experiments of Lloyd and McIntyre, and would appear in those of Alvord and Fuortes, to be the essential factor in randomization of response relative to such monosynaptic

reflex afferent volleys as are presented to the motoneurons. A further important aspect of the experiments of Lloyd and McIntyre for purposes of the present discussion is that the randomization of response relative to the monosynaptic reflex volleys that occurred when long spinal reflex barrage was available to the motoneurons did so at a repetition rate of 25 per minute. Thus, not only does one find that random extraneous bombardment apparently is an essential factor in randomization of response with respect to monosynaptic afferent volleys, but one also finds that temporal summation, by high frequency stimulation, is not essential. There is, therefore, no reason to propose (*cf.* reference 1) that the agency responsible for temporal summation in the monosynaptic reflex pathway is capable by summation during high frequency afferent stimulation of becoming a transmitting agency in its own right. This in turn leads one to conclude that the sustained reflex of Alvord and Fuortes (1) reveals nothing distinctive concerning the reflex mechanism and that such distinctions as have been drawn between sustained reflexes and unsustained reactions are artificial and unjustified.

Another Factor in Depression.—All the frequency dependent variations in mean monosynaptic reflex amplitude of pool responses that have been encountered (11, 6) and most of the behavior of individual motoneurons can be accounted for by the assumption of two processes: (1) subnormality, that limits response at high frequencies; and (2) a process that initially is facilitatory, that reverses at approximately 15 msec. to become depressive, that reaches maximum depressive action at approximately 100 msec., and that then dwindles to insignificance at about 20 seconds. Occasionally an individual motoneuron behaves in a manner that is not completely accounted for by the operation of these two processes. Neuron 4A is one such, also in lesser degree neurons 5 and 6. All three present the same problem.

At a stimulus frequency of 5 per second neuron 4A is depressed to the point that firing index is zero. It remains so depressed at all higher frequencies. Obviously subnormality plays no role in the behavior of neuron 4A since it is completely unresponsive. In the circumstance one would expect neuron 4A to enter the discharge zone by virtue of temporal summation at a stimulus frequency of 100 per second, but it does not. Such a variety of possible explanations for this sort of behavior exists without experimental basis for preference that one can do little more at this time than list some of the more obvious.

1. In this upper range of frequencies influences traceable to group IB or group II may be more effective than other experiments would lead one to suppose (11), or it may be that those influences largely masked in pool responses can be revealed by certain individual units.

2. Although a unit under study may not be responding, and hence subject to subnormality, others about it certainly are. Since the sequelae of discharge by some motoneurons may influence responsivity of others (13) the unit in question may be giving expression to that form of influence.

3. A block may develop in some presynaptic fibers during repetitive stimulation at relatively high frequencies.

SUMMARY

An assemblage of individual motoneurons constituting a synthetic motoneuron pool has been studied from the standpoint of relating monosynaptic reflex responses to frequency of afferent stimulation.

Intensity of low frequency depression is not a simple function of transmitter potentiality.

As frequency of stimulation increases from 3 per minute to 10 per second, low frequency depression increases in magnitude. Between 10 and approximately 60 per second low frequency depression apparently diminishes and subnormality becomes a factor in causing depression. At frequencies above 60 per second temporal summation occurs, but subnormality limits the degree of response attainable by summation.

At low stimulation frequencies rhythm is determined by stimulation frequency. Interruptions of rhythmic firing depend solely upon temporal fluctuation of excitability. At high frequency of stimulation rhythm is determined by subnormality rather than inherent rhythmicity, and excitability fluctuation leads to instability of response rhythm. In short, whatever the stimulation frequency, random excitability fluctuation is the factor disrupting rhythmic response.

Monosynaptic reflex response latency is stable during high frequency stimulation as it is in low frequency stimulation provided a significant extrinsic source of random bombardment is not present. In the presence of powerful random bombardment discharge may become random with respect to monosynaptic afferent excitation provided the latter is feeble. When this occurs it does so equally at low frequency and high frequency. Thus temporal summation is not a necessary factor. There is, then, no remaining evidence to suggest that the agency for temporal summation in the monosynaptic system becomes a transmitting agency in its own right.

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PROTEIN SYNTHESIS IN ISOLATED CELL NUCLEI

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(Received for publication, July 26, 1956)

The present communication deals with the ability of isolated nuclei to incorporate isotopically labelled amino acids into their proteins. It will be shown that the deoxyribonucleic acid of the nucleus plays a role in this incorporation; that protein synthesis virtually ceases when the DNA is removed from the nucleus, and that the uptake of amino acids resumes when the DNA is restored.

This report also deals with the conditions necessary for amino acid incorporation, with the effects of various inhibitors, and with the role of nucleic acid components in this aspect of protein synthesis. Preliminary accounts of some of these experiments have been published elsewhere (1, 2).

Some Properties of Isolated Thymus Nuclei.—Most of the experiments to be described were performed on nuclei isolated from calf thymus tissue in 0.25 M sucrose solution containing a small amount of calcium chloride. The procedure is rapid and simple, and it provides nuclei of high purity in good yield.

Since some form of standardization is essential for all work on isolated cell components, thymus nuclei isolated in sucrose have been compared with those isolated in non-aqueous media (3, 4). The purpose of this comparison is to test whether the aqueous isolation procedure extracts water-soluble nuclear components, or whether it leads to excessive contamination with the water-soluble proteins of the cytoplasm. Many such comparisons have shown that thymus "sucrose" nuclei are the equivalent of the standard "non-aqueous" nuclei in many respects. Their DNA content is the same (2.5 per cent DNA-P), as is their over-all protein composition and enzymic constitution (4).

When preparations of thymus nuclei isolated in sucrose solution are examined under the light microscope, either stained or unstained, they seem to be a beautiful preparation. One observes great numbers of free nuclei, a few whole cells, and occasional red cells. Staining reveals occasional strands of cytoplasm attached to some of the nuclei. The over-all extent of this cytoplasmic contamination can be estimated chemically [*e.g.* by nucleic acid analyses] and the conclusion was reached that these nuclei are better than 90 per cent pure.

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Since many intact thymocytes have only a thin halo or crescent of cytoplasm, the possibility was considered that many objects believed to be free nuclei under the light microscope might be intact cells with scanty or negligible amounts of cytoplasm. To investigate this possibility two preparations

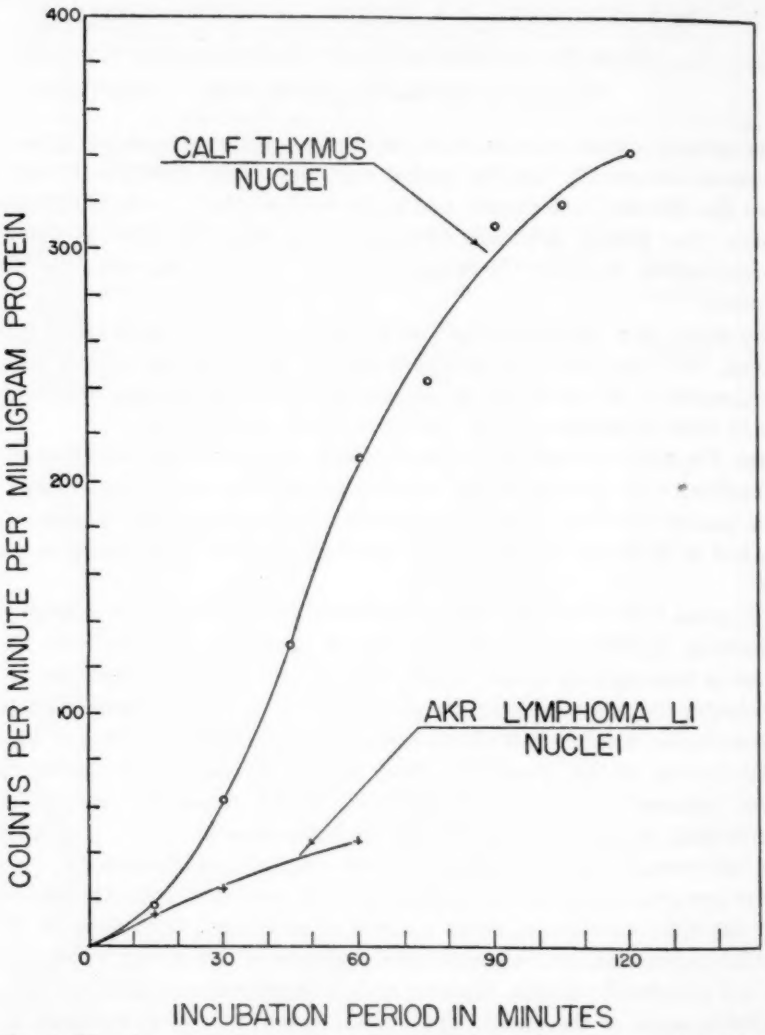

FIG. 1. The time course of alanine-1-C^{14} incorporation into the proteins of isolated nuclei. The specific activity of the total nuclear protein is plotted against the time of incubation of the nuclear suspension. The upper curve shows the incorporation into thymus nuclei. The lower curve shows the rate of alanine-C^{14} uptake by nuclei isolated from an AKR lymphoma.

were examined under the electron microscope. (This was made possible through the generous cooperation of Dr. M. Watson and Dr. G. Palade of The Rockefeller Institute.) Electron microscopy makes it evident that there is some cytoplasmic contamination, but this is also evident in stained preparations under the light microscope. The advantage of the electron microscope in these studies is the ease of detection of small thymocytes. Cell counts on random fields selected from the two preparations gave 45 cells per thousand nuclei in the

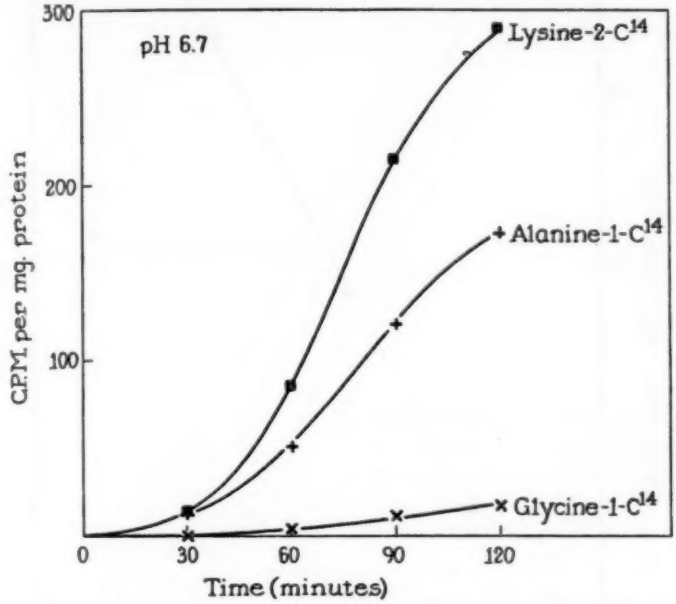

FIG. 2. The time course of incorporation of alanine-1-C^{14}, glycine-1-C^{14}, and lysine-2-C^{14} into the proteins of isolated thymus nuclei. In this plot the data have been "normalized" to show the relative uptakes for equivalent amounts of the different amino acids, when each has the same specific activity in millicuries per millimol.

first preparation and 77 per thousand in the second. Even more convincing evidence for the absence of appreciable whole cell contamination was obtained in studying the effect of deoxyribonuclease. These experiments are described in detail below.

Amino Acid Incorporation into the Proteins of Isolated Nuclei.—When thymus nuclei are suspended in a buffered sucrose medium and incubated at 37°C., in the presence of isotopically labelled amino acids, there is a rapid and considerable incorporation of the isotope into the proteins of the nucleus. Fig. 1 shows the time course of incorporation of alanine-1-C^{14} into the mixed proteins of isolated calf thymus nuclei. The lower curve shows the incorporation

observed into lymphoma nuclei isolated by a similar procedure. (The lymphoma tissue was generously supplied by Dr. John Kidd of Cornell University Medical College.) After an initial "lag" period of about 10 to 15 minutes, the

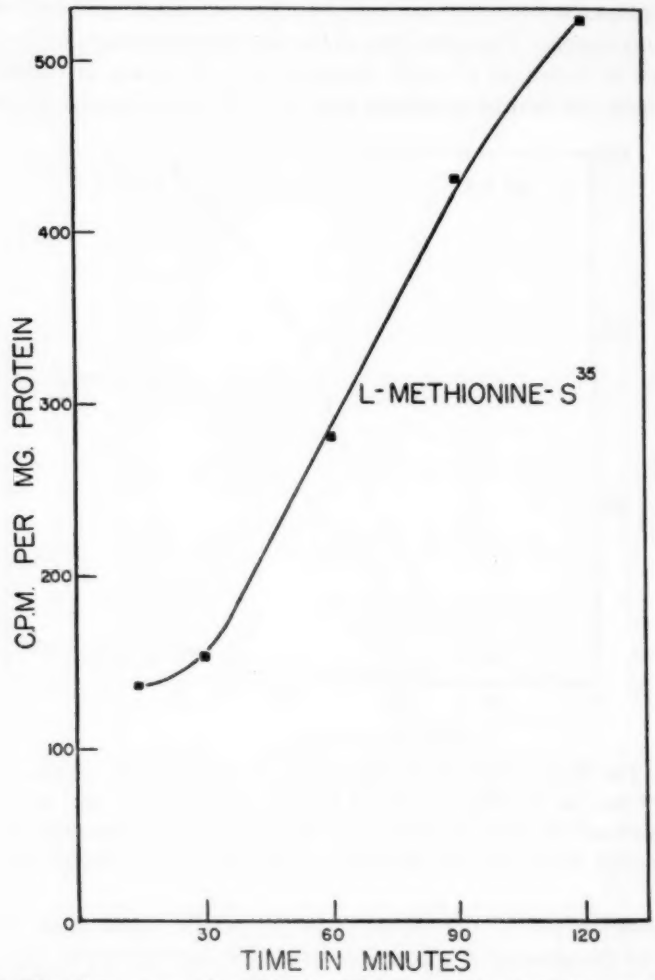

FIG. 3. The time course of methionine-S^{35} incorporation into the proteins of isolated thymus nuclei.

C^{14} uptake proceeds linearly for about 90 minutes and then begins to slowly taper off.

Because a few whole cells are inevitably present in preparations of thymus nuclei, the possibility was considered that the observed amino acid uptake was due to the cells and not to the nuclei. To test this possibility a radioautograph was made of a smear of the nuclear suspension after incubation

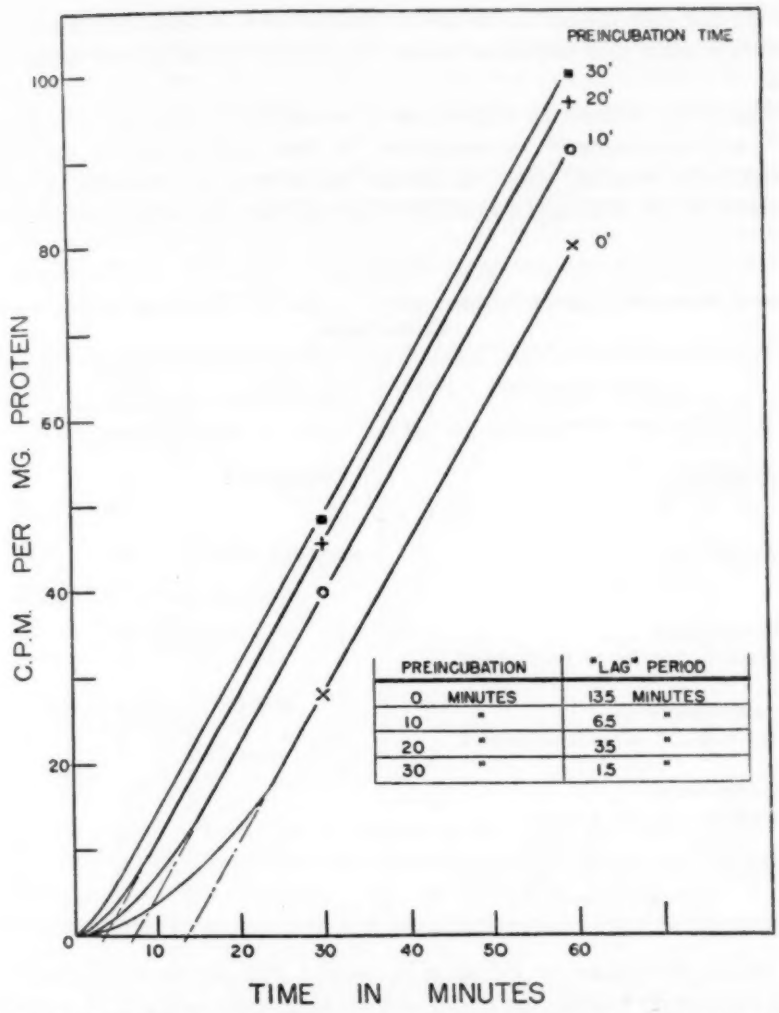

FIG. 4. The effect of preincubation of thymus nuclei upon the subsequent incorporation of alanine-1-C^{14}. The nuclear suspension was preincubated for 10, 20, or 30 minutes before adding the isotopic alanine. The time course of C^{14} incorporation by such preincubated nuclei is compared with that of control nuclei which received the isotope at time zero. Note that preincubation diminishes the lag period without affecting the subsequent rate of C^{14}-alanine incorporation.

with C^{14}-alanine and acid fixation to remove the soluble amino acid. The distribution of silver grains produced in an overlaying photographic emulsion was then examined under the microscope, and it was observed that all components of the suspension were radioactive. The uptake was not simply due

to the few cells present in the smear. Similar work in other laboratories also makes it plain that individual nuclei incorporate the radioactive amino acid (5).

Figs. 2 and 3 show the time course of alanine-1-C^{14}, glycine-1-C^{14}, lysine-2-C^{14}, and methionine-S^{35} incorporations. In these and in most of the experiments to be described below, the uptake was followed by measuring the radioactivity of the total mixed proteins of the nucleus. The results are expressed

TABLE I

Effect of Anoxia and of Several Inhibitors upon C^{14}-Amino Acid Incorporation by Isolated Calf Thymus Nuclei

Conditions of experiment	Isotope administered as	Specific activity of nuclear protein	Degree of inhibition
		C.P.M./mg.	*per cent*
1. Nuclei in O_2	Glycine-1-C^{14}	71	—
" " N_2	"	15	80
2. Nuclei in O_2	DL-Alanine-1-C^{14}	72	—
" " N_2	"	10	85
3. Control nuclei	"	72	—
Nuclei in 2×10^{-4} M dinitrophenol	"	11	84
4. Control nuclei	"	131	—
Nuclei in 2×10^{-4} M Janus green B	"	3	97
5. Control nuclei	"	54	—
Nuclei in 1×10^{-3} M NaCN	"	13	76
" " " " NaN_3	"	5	91
Nuclei + 0.05 mg. dicumarol per ml.	"	8	85
" + 1 μg. antimycin A " "	"	6	89

as counts per minute per milligram of protein. The protein was prepared by first extensively washing the nuclei with trichloroacetic acid (TCA), removing the nucleic acids with hot TCA, and finally removing the lipides with warm ethanol, ethanol-ether-chloroform mixtures, ether, and acetone. The protein residue was then homogenized in acetone and filtered off on filter paper planchets. Radioactivity was determined using a Geiger-Müller tube and scaling circuit, and the measurements were subsequently corrected for self-absorption (6).

To test whether the radioactivity measured was actually in the protein and not merely adsorbed or bound by ester linkages, the protein was treated with alkali and with ninhydrin. In the case of incorporated alanine-1-C^{14}, more than 94 per cent of the activity remained after treatment with 0.25 N NaOH for 2 hours, and more than 89 per cent remained after reaction with

ninhydrin. 76 per cent of the incorporated methionine-S^{35} was stable to alkali. When the different proteins of the nucleus are fractionated before counting, the label remains throughout the fractionation and can be detected in such well defined nuclear components as the "arginine-rich-" and "lysine-rich" histones.

The nature of the lag in the uptake of radioactive alanine into the proteins of the nucleus was investigated by experiments which are summarized in Fig. 4. Nuclei were preincubated at 37° for 10, 20, or 30 minutes before adding the isotopic alanine. The time course of C^{14} incorporation by such preincubated

TABLE II

Effect of Fluoride and of Added Metabolites upon C^{14}-Amino Acid Incorporation by Isolated Calf Thymus Nuclei

Conditions of experiment	Isotope administered as	Specific activity of protein
		c.p.m./mg.
1. Control nuclei	DL-Alanine-1-C^{14}	62
Nuclei in 1×10^{-3} M Na F	"	57
2. Nuclei without added metabolite	"	40
" + 40 μM glucose	"	44
" " " " fructose	"	40
3. Nuclei without added metabolite	Glycine-1-C^{14}	86
" + 40 μM glucose	"	99
" " " " α-ketoglutarate	"	109

nuclei was compared with that of control nuclei which had received the isotope at time zero. It is evident that preincubation diminishes the lag period without affecting the subsequent rate of C^{14}-alanine incorporation. These findings suggest that an activation of some sort occurs during preincubation. Further evidence for such activation will be presented below in connection with the inhibitory effects of benzimidazole derivatives. [The possibility was also considered that the lag in C^{14}-alanine uptake might be due to the existence of a "fixed pool" of unlabelled alanine in the nuclei, a pool which must be depleted before isotopic alanine is taken up. This would be analogous to the interesting situation described by Cowie and Walton (7) of fixed amino acid pools in yeast. There is, however, a fundamental distinction between the two systems. In their experiments labelled amino acids in the pool went directly to protein, and the rate of this incorporation was not affected by adding C^{12}-amino acids to the medium. Similar experiments with isolated thymus nuclei have shown that exogenous, unlabelled L-alanine is in rapid and free exchange with the C^{14}-alanine of the pool.]

There are a number of experiments now to be described which deal with

the conditions necessary for amino acid incorporation, and with the inhibition of nuclear protein synthesis.

The uptake of amino acids is an aerobic phenomenon which does not occur in a nitrogen atmosphere and which is inhibited by a number of substances

TABLE III

Effect of Chloramphenicol, Cortisone, and Amino Acid Antagonists upon C^{14}-Amino Acid Incorporation by Isolated Calf Thymus Nuclei

Conditions of experiment	Isotope administered as	Specific activity of nuclear protein	Change in C^{14} or S^{35} uptake
		C.P.M./mg.	*per cent*
1. Control nuclei	DL-Alanine-1-C^{14}	97	—
Nuclei in 3.2×10^{-4} M chloramphenicol	"	97	0
2. Control nuclei	Glycine-1-C^{14}	21	—
Nuclei in 1.6×10^{-4} M chloramphenicol	"	19	−10
3. Control nuclei	L-Methionine-S^{35}	137	—
Nuclei in 1.6×10^{-4} M chloramphenicol	"	136	0
4. Control nuclei	DL-Alanine-1-C^{14}	43	—
Nuclei in 1×10^{-3} M *p*-fluorophenylalanine	"	37	−14
5. Control nuclei	"	37	—
Nuclei in 5×10^{-3} M *p*-fluorophenylalanine	"	31	−15
6. Control nuclei	"	65	—
Nuclei in 1.5×10^{-4} M ethionine	"	56	−14
" " " " 10^{-3} " "	"	51	−21
7. Control nuclei	DL-Lysine-2-C^{14}	23	—
Nuclei in 1.5×10^{-4} M ethionine	"	22	−4
" " " " 10^{-3} " "	"	22	−4
8. Control nuclei	L-Methionine-S^{35}	221	—
Nuclei in 1.5×10^{-4} M ethionine	"	215	−3
" " " " 10^{-3} " "	"	194	−12
9. Control nuclei	DL-Alanine-1-C^{14}	217	—
Nuclei + 0.063 mg. cortisone	"	138	−36
" " 0.125 " "	"	122	−54

which are known to block oxidative phosphorylation (Table I). Cyanide, azide, dinitrophenol, Janus green b, dicumarol, and antimycin A all inhibit alanine uptake. However, methylene blue, a substance which inhibits oxidative phosphorylation in mitochondria, has only a small effect on nuclear isotope

incorporation. The nature of the oxidative phosphorylation in preparations of isolated thymus nuclei is discussed in detail in the following paper.

Adding metabolites, such as glucose, fructose, or α-ketoglutarate to the nuclear suspension has little effect on the incorporation of amino acids (Table II). Added glucose may at times raise the alanine uptake by about 10 per cent. An attempt to block glycolysis by the addition of fluoride did not result in inhibition of alanine incorporation.

It was shown by Gale and Folkes that chloramphenicol blocks protein synthesis in bacteria (8). This substance has little effect on glycine-1-C^{14}, alanine-1-C^{14}, or methionine-S^{35} uptake by isolated nuclei (Table III). The

TABLE IV

Effect of Benzimidazole Derivatives on Alanine-1-C^{14} Uptake by Isolated Nuclei

Derivative tested		Amount	Specific activity of nuclear protein	Inhibition of uptake	Relative inhibition of virus multiplication*
		mg.	*C·P·M./mg.*	*per cent*	
Control		—	50	—	—
5,6-Dichlorobenzimidazole	1-β-D Arabinopyranoside	0.1	45	10	3.1
"	1-β-D-Ribopyranoside	0.1	36	28	15
"	1-β-D-Ribofuranoside	0.1	26	48	92
4,5,6-Trichlorobenzimidazole	1-α-D-Ribofuranoside	0.01	46	8	165
"	1-β-D-Ribofuranoside	0.01	36	28	760

* Work of Tamm *et al.* (11–13).

table also summarizes some experiments using *p*-fluorophenylalanine, an amino acid antagonist, which was shown by Halvorson and Spiegelman to block protein synthesis in bacteria (9). We were unable to test this compound at the higher concentrations used in Halvorson's experiments, but at 5×10^{-3} M it inhibited alanine uptake by only 15 per cent. Similarly, added ethionine (1.5×10^{-4} M) had no effect on the incorporation of methionine-S^{35} or lysine-2-C^{14}, and it reduced alanine uptake by only 14 per cent.

In considering possible inhibitors of nuclear protein synthesis, our attention was directed to the remarkable observation that repeated injections of cortisone lead to involution of the thymus gland (10). When tested on thymus nuclei, *in vitro*, small amounts of cortisone lead to greatly reduced alanine-C^{14} uptakes (Table III).

Among the inhibitors tested, a series of substituted benzimidazoles was of greatest interest. Typical of this class of compounds is the substance 5,6-

dichloro-β-D-ribofuranosylbenzimidazole (DRB). This structural analog of a purine riboside was found by Tamm *et al.* to retard influenza virus multiplication in the mouse and in tissue culture (11–13). When tested on isolated thymus nuclei it effectively inhibits the uptake of alanine-1-C^{14}. Tests of this

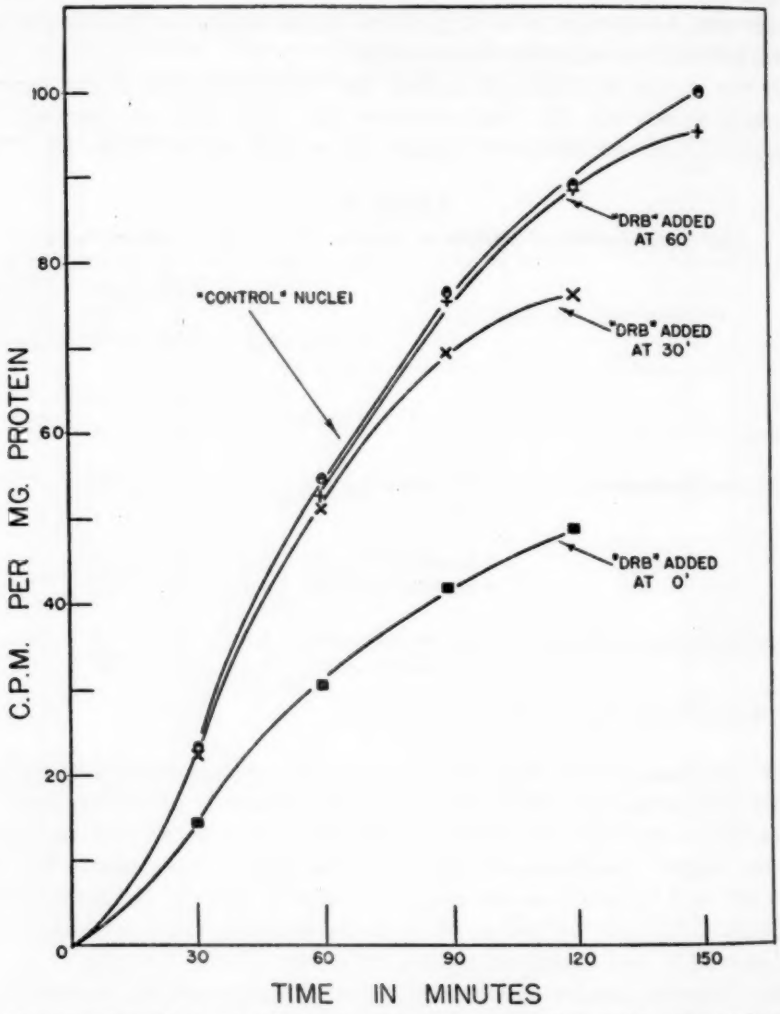

FIG. 5. The effect of 5,6-dichloro-β-D-ribofuranosylbenzimidazole (DRB) on alanine-1-C^{14} incorporation into the proteins of isolated thymus nuclei. The time course of C^{14} incorporation in the presence of DRB is compared with that observed in control nuclei (upper curve). Note that DRB added together with the isotopic alanine (at time zero) is an effective inhibitor, whereas DRB added 30 or 60 minutes later is far less effective.

and similar compounds (synthesized by C. Shunk and K. Folkers of the Merck Research Laboratories) are summarized in Table IV. It is of special interest that the inhibitions observed in the isolated nuclei closely parallel those found in tests of viral growth. The ribosyl benzimidazole is an effective inhibitor while the corresponding arabinose derivative is not. Effective inhibition also requires that the ribose exist in the furanose, not the pyranose form, and

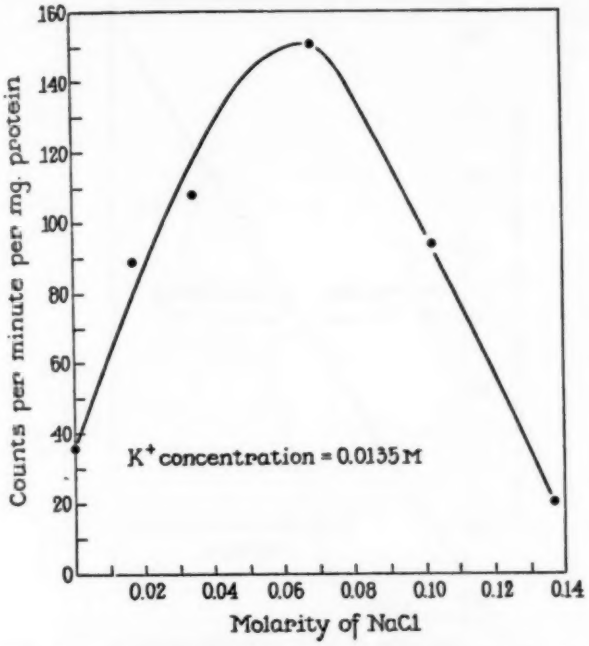

FIG. 6. The effect of varying the sodium ion concentration of the incubation medium on the ability of thymus nuclei to incorporate glycine-1-C^{14}. The specific activity of the nuclear protein after 60 minutes' incubation of the nuclei in the presence of glycine-C^{14} is plotted against the sodium concentration of the medium.

that the glycosidic linkage between the sugar and the benzimidazole ring should have the β-, and not the α-, configuration.

A remarkable feature of these inhibitors in the viral growth system is the limitation of their time of action. They effectively retard virus multiplication only if they are present at the time of infection or shortly afterward, and much of their effectiveness is lost if they are administered several hours after infection has occurred. This limitation on the time of action is also evident when benzimidazole derivatives are tested on isolated thymus nuclei. The experiments summarized in Fig. 5 show that when 5,6-dichloro-β-D-ribofuranosylbenzimidazole (DRB) is added together with the isotopic alanine, the

uptake is inhibited from the outset, and for the duration of the experiment. However, when the DRB is added 30 or 60 minutes after adding the C^{14}-alanine there is no appreciable inhibition of the amino acid uptake. The findings suggest that DRB interferes with a preliminary "activation" of nuclear pro-

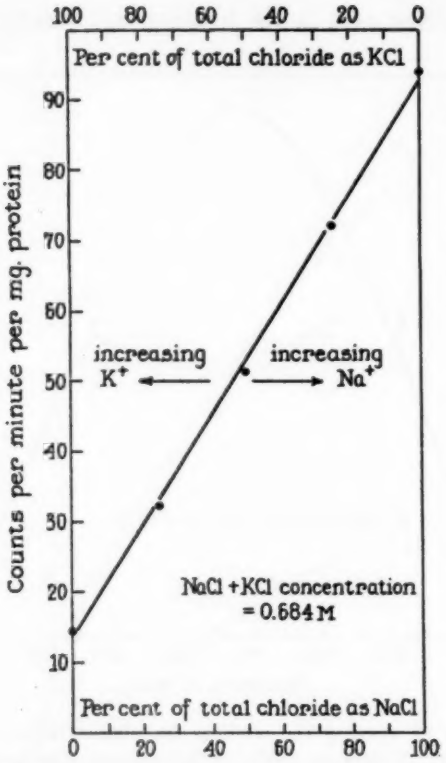

FIG. 7. The effect of varying sodium/potassium ratio on glycine-1-C^{14} incorporation by isolated thymus nuclei. The specific activity of the nuclear protein after 60 minutes' incubation is plotted against the ratio of sodium to potassium ions in the medium, the total salt concentration being held constant.

tein synthesis, and that once this activation has occurred the inhibitor is no longer effective. This agrees with the conclusions drawn previously from the study of the lag phase in alanine-1-C^{14} incorporation. Evidence is presented below to suggest that DRB inhibits the synthesis of protein by interfering with a preliminary synthesis of ribonucleic acid.

One of the most striking effects on nuclear amino acid incorporation is that produced by sodium ions. Nuclei isolated in sucrose need a sodium supplement in order to incorporate amino acids actively. Fig. 6 shows the effect of varied

sodium concentration on the level of glycine-1-C^{14} uptake. There is a well defined maximum at about 0.07 M under our test conditions. The sodium requirement seems to be specific. An attempt was made to substitute all or part of the sodium in the medium with an equivalent amount of potassium. In these experiments the total salt concentration was kept constant and the

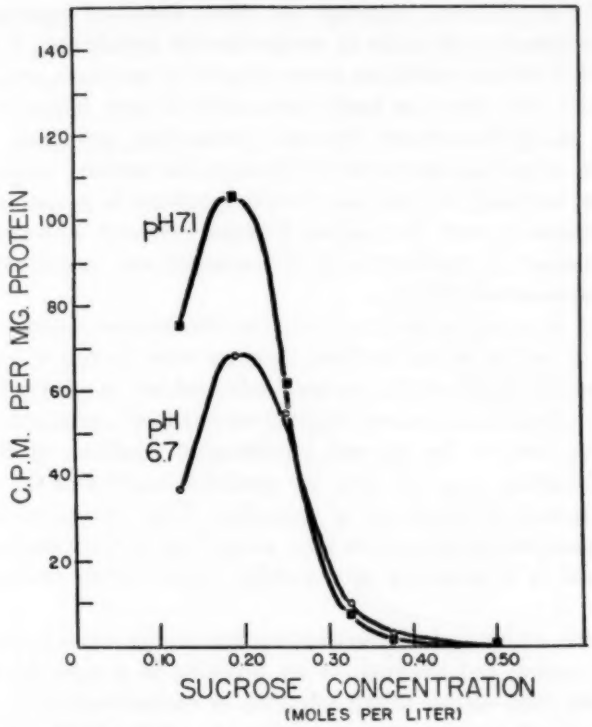

FIG. 8. The effect of varying the sucrose concentration of the medium upon the ability of thymus nuclei to incorporate alanine-1-C^{14}. The specific activity of the nuclear protein after 60 minutes' incubation is plotted against the sucrose concentration of the incubation medium.

ratio NaCl/KCl was varied. When all the sodium is replaced by potassium the uptake falls to 15 per cent of the optimal value. Increasing the sodium to potassium ratio gives a corresponding increase in the amount of amino acid incorporated (Fig. 7). When the sodium concentration is optimal, the addition of small amounts of potassium (chloride) does not influence the uptake.

The extreme dependence of the nucleus' capacity to incorporate amino acids upon sodium ion concentration makes it necessary to control rather rigorously the sodium level of the incubation medium. This introduces a related problem,

namely the osmotic balance between nuclear structures and the suspending medium. It was soon found that nuclei exposed to high sucrose concentrations lost their ability to incorporate amino acids into protein. A more detailed study of this dependence of uptake upon sucrose concentration is presented in Fig. 8. The peak of activity under our test conditions occurs at about 0.20 M. The medium also contains sodium phosphate buffer (0.025 M), glucose (0.02 M), and NaCl (0.03 M). Although the effects described bear some resemblance to the osmotic properties of semipermeable membranes, it should be pointed out that thymus nuclei are not enveloped by an intact, semipermeable membrane, and that they are freely permeable to large molecules, such as ribonuclease, deoxyribonuclease, histones, protamines, and basic dyes. The marked effects of sucrose concentration upon nuclear activity suggest that the fine structure necessary for nuclear protein synthesis is in osmotic balance with its environment, and that nuclear function can vary with that balance. This is reminiscent of experiments on the extended and contracted states of interphase chromosomes (14).

Although it is a simple matter to control the sucrose concentration and sodium level of the incubation medium, there are other factors which influence nuclear amino acid uptake which are not readily subject to control. In working with tissue obtained from commercial packers one finds variations in the age of the thymus, and in the sex and physiological condition of the animal. Weather and feeding, too, can vary the synthetic activity of the nucleus in ways which cannot be predicted or controlled. Thus the actual amount of amino acid incorporated in a given time varies from one nuclear preparation to another, and it is necessary to run daily controls when comparisons are being made.

The synthetic activity of the isolated nucleus can be readily destroyed by heating, by freezing and thawing, or by breaking in a high-speed blendor. Nuclei removed from sucrose to salt solutions, or washed with 0.1 M phosphate buffer prior to incubation in a sucrose medium, lose their ability to incorporate amino acids. Nuclei stored at 2°C. gradually lose their synthetic activity. The rate of decline in activity with storage varies considerably in different preparations, but in most cases the ability to incorporate amino acids into nuclear protein is well retained for 4 to 6 hours and is almost entirely lost after 24 hours at 2°. Nuclei held at 37°C. lose their synthetic capacity more rapidly. The rate of C^{14}-alanine uptake may be cut in half after 1 hour at 37°, and be reduced to 25 per cent of the original value after 2 hours.

Synthesis vs. "Exchange".—There are several experiments now to be described which make it clear that the uptake of labelled amino acids into nuclear proteins is specific for L-amino acids and that it represents an essential aspect of protein synthesis, and is not simply a random, non-specific exchange reaction involving a few labile groups of the nucleus.

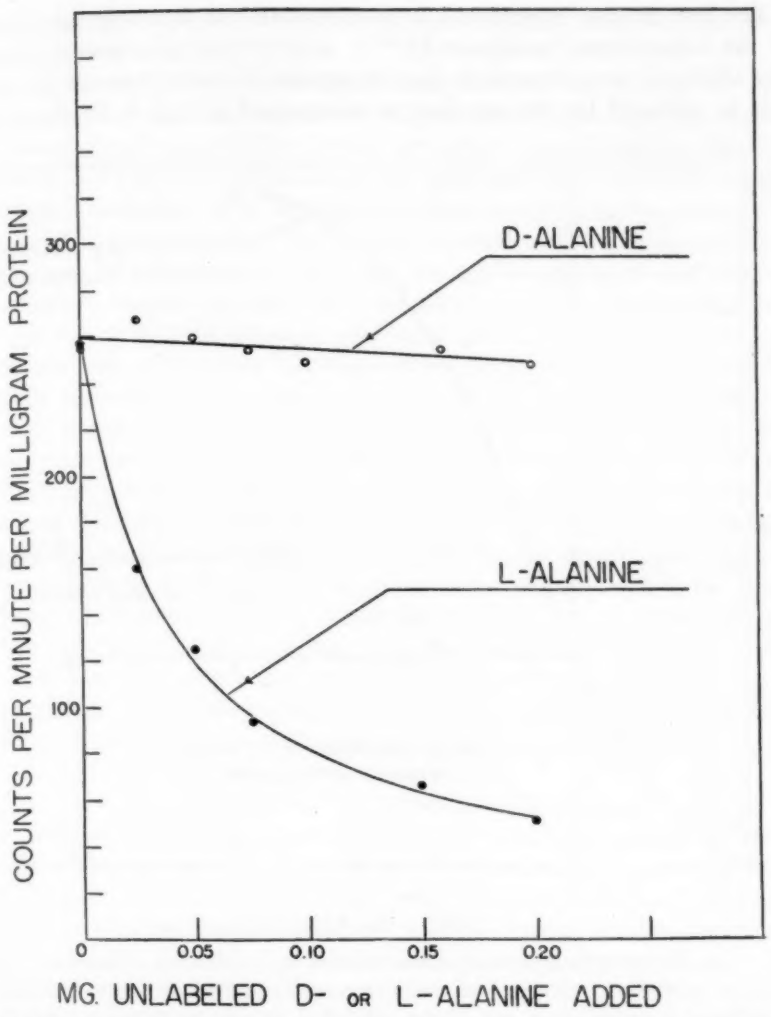

FIG. 9. The effect of unlabelled D- and L-alanines upon incorporation of DL-alanine-1-C^{14} by isolated thymus nuclei. The specific activity of the nuclear protein after 60 minutes' incubation is plotted against the amount of unlabelled D- or L-alanine added to the medium. Note that added D-alanine has no effect on C^{14}-uptake, whereas L-alanine competes with isotopic molecules for acceptance by the nucleus. It follows that only the L-isomer of the administered C^{14}-alanine is utilized for nuclear protein synthesis.

The first of these experiments is the demonstration that only the L-isomer of the administered DL-alanine-1-C^{14} is incorporated into nuclear protein. The ability of the nucleus to distinguish between D- and L- forms of the amino acid is indicated by the experiments summarized in Fig. 9. In these tests

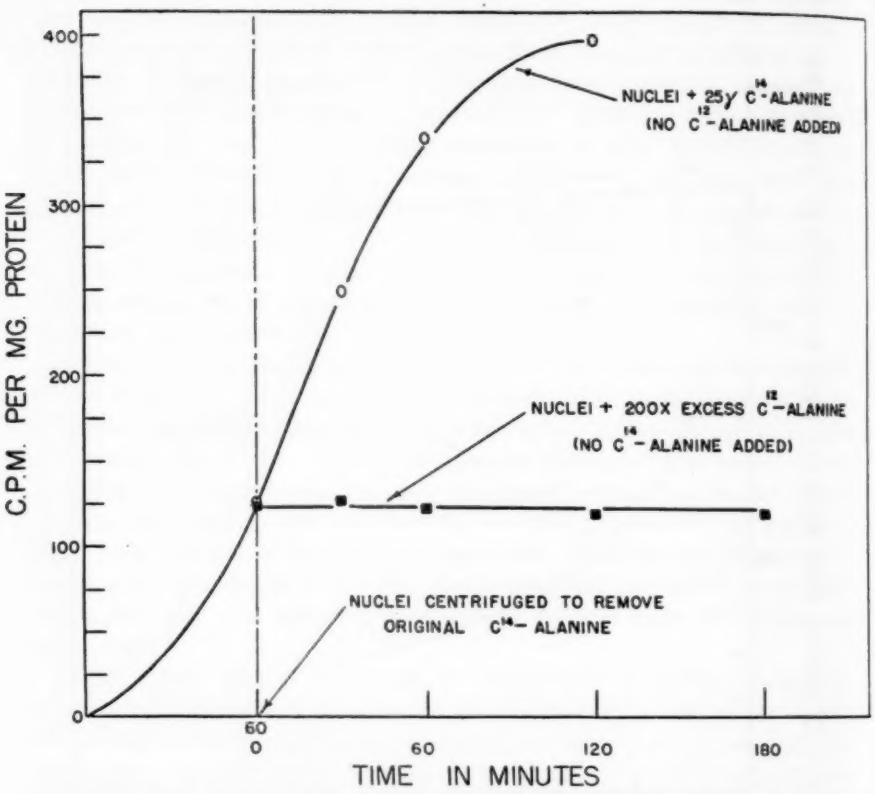

FIG. 10. The retention of incorporated alanine-1-C^{14} following addition of a large excess of unlabelled L-alanine (see text). The specific activity of the nuclear protein at different intervals after adding the unlabelled alanine is plotted against time. The upper curve shows that the nuclei in these experiments are still able to incorporate C^{14}-amino acid.

increasing amounts of unlabelled D-alanine or unlabelled L-alanine were added to the incubation medium together with the DL-alanine-1-C^{14}. If both the D- and L- forms of the C^{14}-alanine were utilized by the nucleus then both unlabelled isomers should compete with isotopic molecules for acceptance by the nucleus, and the C^{14}-activity of the nuclear protein should be correspondingly lowered. Actually, the addition of unlabelled D-alanine has no such effect; only the L-isomer competes for incorporation. It follows, therefore,

that only the L-form of the C^{14}-alanine administered is utilized for protein synthesis by the nucleus.

A second experiment was designed to test whether amino acid once incorporated is in constant exchange with other amino acid molecules in the medium. If the uptake of amino acids is indeed reversible then C^{14}-alanine once incorporated into nuclear proteins should be exchanged with unlabelled L-alanine added to the medium after incorporation has occurred. This was tested in the experiments summarized in Fig. 10. Incorporation of C^{14}-alanine was allowed to proceed for 60 minutes. At that time the nuclei were centrifuged down and the isotopic alanine was removed. The nuclei were then resuspended in the presence of a 200-fold excess of unlabelled L-alanine. Samples of the nuclei were removed at 30 minute intervals and the carbon-14 activity of the nuclear protein measured in the usual way. It is evident from the curves that the specific activity of the nuclear proteins remains essentially constant for 3 hours even in the presence of a great excess of unlabelled alanine. It follows that the C^{14}-alanine incorporated into nuclear protein is not subject to selective replacement by unlabelled alanine in the medium. The figure also shows that when additional C^{14}-alanine is added to the nuclei in such experiments, incorporation proceeds in the usual way. Thus the nuclei will continue to incorporate C^{14}-amino acid under conditions where they will not give it up. Similar experiments have been performed using S^{35}-labelled methionine and parallel results were obtained. It follows that the incorporation of isotopic amino acids into the proteins of isolated nuclei is essentially irreversible, and the possibility can be excluded that a few active centers in the nuclear protein are in constant amino acid exchange with the medium.

(It should be noted, however, that experiments of this kind do not exclude the possibility of a slow, random "turnover" involving a large number of amino acid residues, all equally subject to replacement; because if a great number of the alanine or methionine residues in the nucleus are able to exchange, and only a few of these turn over in the 3 or 4 hour experimental period, then the rate of loss of C^{14}-alanine or S^{35}-methionine once incorporated will be too small to be followed experimentally.)

It may be of value at this point to clarify our usage of the term "protein synthesis." In all experiments in which one measures the incorporation of labelled amino acids into proteins, the question naturally arises as to the relationship between the uptake observed and the synthetic process. In the strictest sense "synthesis" can be applied only in cases in which a net production of new protein molecules can be demonstrated. When such synthesis occurs in the presence of isotopically labelled amino acids the protein formed will be labelled. However, the incorporation of isotopic amino acids does not, in itself, constitute a proof that new protein molecules have been synthesized. Although in these experiments on isolated nuclei, a net synthesis of new protein has not been directly demonstrated, a number of observations make it most prob-

able that we are dealing with a very direct aspect of the synthetic mechanism: (1) The uptake of amino acids is energy-dependent. (2) The amount of amino acid incorporated into definite proteins increases with time. When the proteins of the nucleus are fractionated before counting (see below), one observes a progressive time course of amino acid uptake into such well defined protein fractions as the arginine- and lysine-rich histones. (3) The relative rates of amino acid uptake into different nuclear proteins *in vitro* are in accord with the rates observed in *in vivo* experiments. (4) Isotopic amino acid molecules once incorporated are not rapidly lost by exchange with unlabelled molecules in the medium.

From the amount of isotopic alanine incorporated an estimate can be made of the amount of new protein which may be formed. In a typical experiment 30 mg. of nuclei (10^9 nuclei) incorporate 0.6 μg. (2.4 per cent) of the administered C^{14}-alanine in 1 hour. If the average nuclear protein contains about 7.6 per cent alanine (15), this uptake corresponds to the formation of about 8 μg. of protein per hour, an amount too small to be demonstrated directly by methods now available to us. Nevertheless, the extent of the synthesis can be better appreciated from the calculation that each nucleus synthesizes 22 molecules of protein of average molecular weight 50,000 every second.

Attempts have been made to increase the extent of incorporation of isotopic alanine and glycine by the addition of a supplement of mixed L-amino acids to the nuclear suspension. No stimulation was observed, which leads to the conclusion that, if the uptake of labelled amino acids represents a net synthesis of protein, the other amino acids required must be present in the nuclei. That this is indeed the case for thymus nuclei isolated by the Behrens' procedure has been recently shown by Kay (16).

The Role of Deoxyribonucleic Acid in Protein Synthesis.—The notion originally suggested by Brachet and by Caspersson that ribonucleic acids play a role in protein synthesis is now widely accepted as demonstrated. The most direct evidence in its favor stems from the work of Gale and Folkes on amino acid uptake by bacterial cell residues (17), and from work in this laboratory on amino acid incorporation by a ribonucleoprotein complex of the liver (18). In both cases ribonuclease acts to suppress amino acid incorporation.

The isolated cell nucleus affords a unique opportunity to test the role of the deoxyribonucleic acids in the process of protein synthesis, and the results have a special interest because they ultimately bear on the mode of action of the gene, and on the chemical relationships between the nucleus and the cytoplasm.

A number of experiments which relate deoxyribonucleic acid to the process of protein synthesis in the nucleus will now be described. Several of these experiments deal with the effect of deoxyribonuclease on the uptake of labelled amino acids. When isolated nuclei are treated with crystalline pancreatic

deoxyribonuclease before adding isotopic amino acids, the incorporation of the latter is markedly impaired. The degree of impairment becomes more serious as more and more of the deoxyribonucleic acid is removed. Experiments showing the relationship between loss of DNA and inhibition of alanine-1-C^{14} and lysine-2-C^{14} uptakes are summarized in Fig. 11. At the outset there

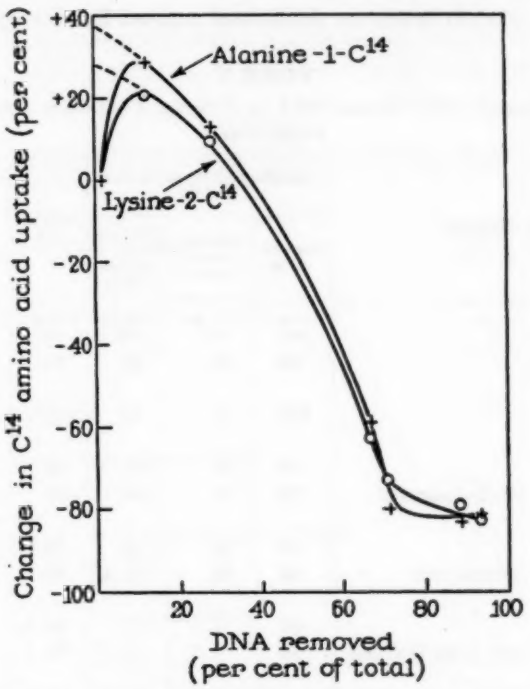

FIG. 11. The effect of removing DNA from thymus nuclei upon the subsequent incorporation of alanine-1-C^{14} and lysine-2-C^{14}. Nuclei were pretreated with DNAase to remove increasing amounts of their DNA. They were subsequently incubated for 60 minutes in the presence of the isotopic amino acid. The decrease in specific activity of the nuclear protein in treated nuclei, relative to that observed in untreated, control nuclei, is plotted against the per cent removal of the DNA.

is roughly a 1:1 correspondence between the per cent DNA removed and the per cent inhibition of the uptake. The inhibition becomes more marked when 30 to 80 per cent of the DNA is removed. Beyond that point the loss of DNA has little effect on the uptake, which remains constant at about 15 to 20 per cent of that observed in control experiments. This sensitivity of the nucleus to treatment with deoxyribonuclease is further evidence for the absence of appreciable whole cell contamination, because intact cells are not

sensitive to treatment with this enzyme. Thymus tissue slices or minces, for example, show only a very slight inhibition of alanine-1-C^{14} uptake following treatment with deoxyribonuclease, and the inhibition observed (about 15 per cent) could easily be attributed to enzyme attack of cells which had been damaged mechanically.

The foregoing experiments suggest that deoxyribonucleic acid plays a vital role in nuclear protein synthesis. Additional support for this conclusion arises

TABLE V

Effect of Supplementary Calf Thymus DNA in Restoring C^{14}-Alanine Uptake in DNAase-treated Nuclei

Supplement tested	Specific activity of nuclear protein			Activity lost	Activity regained	Recovery of lost activity
	Control nuclei	DNAase-treated nuclei	Treated nuclei + supplement			
	C·P·M·/mg.	*C·P·M·/mg.*	*C·P·M·/mg.*	*C·P·M·/mg.*	*C·P·M·/mg.*	*per cent*
1. Thymus DNA	99	15	55	84	40	48
2. " "	135	21	93	114	72	64
3. " "	99	33	101	66	68	103
" " alkali-denatured	99	33	104	66	71	108
4. Thymus DNA	40	10	29	30	19	63
" " apurinic acid	40	10	17.4	30	7.4	25
5. Thymus DNA	38	7.5	28	30.5	20.5	67
" " core after DNAase digestion	38	7.5	24.5	30.5	17	56
Thymus DNA dialyzable products of DNAase digestion	38	7.5	29	30.5	21.5	70

from experiments which test whether the synthetic activity of nuclei treated with deoxyribonuclease can be restored by the addition of supplementary DNA. In these experiments the nuclei were treated with deoxyribonuclease until 70 per cent or more of the DNA had been released into the medium. The nuclei were then centrifuged down, and the medium containing the DNA hydrolysis products was removed. The nuclei were then resuspended in the presence of a calf thymus DNA preparation: isotopic amino acid was supplied and incorporation allowed to proceed for 60 minutes. The specific activity of the protein in nuclei which had received the deoxyribonucleic acid supplement was then compared with the protein activities in control nuclei which had been treated with the enzyme in the same way but which had not received

any additional deoxyribonucleic acid. The data in Table V make it clear that added calf thymus DNA does, in fact, restore much, and at times, nearly all the original activity of the nucleus. Other experiments were performed to measure the amount of added nucleic acid taken up by DNAase-treated nuclei (Table VI). The results show that under the conditions used in all the "restoration" experiments, 30 to 40 per cent of a 5 mg. DNA supplement enters the nuclei.

It is a matter of some interest that when thymus nuclei in sucrose solution are treated with DNAase, they lose 90 per cent of their DNA and yet no more

TABLE VI

Combination of Nucleic Acids with DNAase-Treated Nuclei

Conditions of experiment	Amount of DNA in nuclei	Amount of added DNA combined	Per cent of added DNA combined
	mg.	*mg.*	
Control nuclei	11.2	—	—
Nuclei treated with DNAase	2.6	—	—
DNAase-treated nuclei + 5 mg. thymus DNA	4.13	1.63	33
" " + 10 " " "	4.6	2.0	20
DNAase-treated nuclei + 5 mg. alkali-denatured DNA	4.5	1.9	38
" " + 10 " " "	5.0	2.4	24
" " + 5 mg. yeast RNA	4.3	1.7	34
" " + 10 " " "	5.6	3.0	30

than 15 per cent of the histone is released, although histones are water-soluble and known to be attached to the DNA in the nucleus. This contrasts strongly with the properties of the same nuclei in a saline medium, for when DNA is removed and sucrose is not present, half of the histone is released from the nuclei.

This ability of the thymus nucleus in sucrose to retain its histones despite the removal of DNA may account for the ease with which added DNA is taken up by DNAase-treated nuclei, and it suggests that the proteins of the chromosome are still arranged in a specific and functional configuration.

Pretreatment of isolated thymus nuclei with deoxyribonuclease affects the uptake of different amino acids to different extents (Table VII). Methionine-S^{35} incorporation, for example, was reduced by only 42 per cent under conditions where alanine-1-C^{14} uptake was inhibited 75 per cent. But, despite this

difference, the addition of a DNA supplement seems to restore the lost activity equally in both cases.

Several experiments were performed to test whether chemically or physically modified DNA could restore the uptake in DNAase-treated nuclei. It was found that DNA denatured by treatment with alkali (pH 12.2 for 16 hours (19)) was just as effective as the original DNA preparation. Apurinic acid, while not as effective as intact DNA, does produce an appreciable stimulation of uptake in the DNAase-treated nuclei. The non-dialyzable DNA "core" which remains after exhaustive digestion of thymus DNA with deoxyribo-

TABLE VII

Effect of DNAase Treatment on Alanine-1-C^{14} and Methionine-S^{35} Incorporation by Isolated Nuclei

Conditions of experiment	Isotope administered as	Specific activity of nuclear protein	DNAase / control	Activity lost	Activity regained	Recovery of lost activity
		c.p.m./mg.	*per cent*	*c.p.m./mg.*	*c.p.m./mg.*	*per cent*
Control nuclei	DL-Alanine-1-C^{14}	40	—	—	—	—
Nuclei pretreated with DNAase	"	10	25	30	—	—
DNAase-treated nuclei + thymus DNA	"	29	—	30	19	63
Control nuclei	L-Methionine-S^{35}	141	—	—	—	—
Nuclei pretreated with DNAase	"	82	58	59	—	—
DNAase-treated nuclei + thymus DNA	"	119	—	59	37	63

nuclease (20), and the dialyzable split products of the digestion are both able to replace the "whole" DNA in this system (Table V).

Since even degraded thymus DNA can function in restoring amino acid uptake, it was also a matter of considerable interest to test whether nucleic acids other than calf thymus deoxyribonucleic acid can restore the synthetic capacity of DNAase-treated nuclei. We first compared calf thymus DNA with the DNA of wheat germ and found the latter to be far less effective than the former. However, when the study was extended to include DNAs from other sources, it was found that all the other nucleic acids tested were quite comparable to thymus DNA in their ability to restore C^{14}-amino acid incorporation. A number of such experiments are summarized in Table VIII. Even sea urchin sperm DNA was as effective as the DNA of the thymus.

The next step was to test the effect of ribonucleic acids on amino acid uptake by DNAase-treated nuclei. Two preparations were tested, one from calf liver,

and the other from yeast. Both were just as effective as the DNA of the thymus (Table IX). Since RNA could replace DNA in this system an attempt was made to substitute a mixture of mononucleotides for the RNA. This mixture was made up of the nucleoside 2′ or 3′ phosphates in the same proportions in which they occur in yeast RNA. These did not restore uptake. By the same token, RNA hydrolyzed in dilute alkali to yield the mononucleotides could not restore alanine incorporation in DNAase-treated nuclei.

Other compounds tested without effect included AMP, ADP, and ATP, and mixtures of the purine and pyrimidine bases. Several dinucleotides pre-

TABLE VIII

Effect of Different DNAs in Restoring C^{14}-Alanine Uptake in DNAase-treated Nuclei

Supplement tested	Specific activity of nuclear protein			Activity lost	Activity regained	Recovery of lost activity
	Control nuclei	DNAase-treated nuclei	Treated nuclei + supplement			
	C·P·M./mg.	*C·P·M./mg.*	*C·P·M./mg.*	*C·P·M./mg.*	*C·P·M./mg.*	*per cent*
1. Thymus DNA	145	69	101	76	32	42
Wheat germ DNA	145	69	86	76	17	22
2. Thymus DNA	91	25	70	66	45	68
Calf kidney DNA	91	25	70	66	45	68
Chicken erythrocyte DNA	91	25	60	66	35	53
Paracentrotus sperm DNA	91	25	72	66	47	71
3. Thymus DNA	135	21	93	114	72	63
Trout sperm DNA	135	21	89	114	68	60

pared from yeast RNA were made available to us through the generosity of Dr. R. B. Merrifield of The Rockefeller Institute. These included adenylic-adenylic dinucleotide, adenylic-guanylic dinucleotide, adenylic-uridylic dinucleotide, and guanylic-cytidylic dinucleotide. None of these compounds could restore uptake in DNAase-treated nuclei.

Although fragments as small as dinucleotides do not seem to be active in our system (with the reservation that only a few of the possible dinucleotides were tested), it was found that the dialyzable split products of RNA digestion by RNAase were partially effective; and it has already been pointed out that the dialyzable split products of DNA digestion by DNAase were quite effective. Both these findings suggest that relatively simple, low molecular weight compounds, perhaps of the order of trinucleotides, are able to substitute for DNA in mediating or facilitating amino acid incorporation.

Further fractionation studies are now in progress in an attempt to further characterize and isolate the active dialyzable material.

That intact nucleic acids have a high degree of specificity in biological systems is evident both from the role of DNA in bacterial transformation (21), and of RNA in tobacco mosaic virus infection (22–24). In the amino

TABLE IX

Effect of Ribonucleic Acids and Ribonucleotides on C^{14}-Alanine Uptake by DNAase-treated Nuclei

Supplement tested	Specific activity of nuclear protein			Activity lost	Activity regained	Recovery of lost activity
	Control nuclei	DNAase-treated nuclei	Treated nuclei + supplement			
	C.P.M./mg.	*C.P.M./mg.*	*C.P.M./mg.*	*C.P.M./mg.*	*C.P.M./mg.*	*per cent*
1. Calf liver RNA	92	54	80	38	26	68
" thymus DNA	92	54	81	38	27	71
2. Thymus DNA	68	23	54	45	31	69
Yeast RNA	68	23	58	45	35	78
" " hydrolyzed in 0.3 N NaOH	68	23	23	45	0	0
Yeast RNA dialyzable after RNAase digestion	68	23	36	45	13	29
A, G, U, C, mononucleotide mixture	68	23	23	45	0	0
3. Thymus DNA	83	14	46	69	32	46
Yeast RNA	83	14	45	69	31	45
Adenylic-adenylic dinucleotide	83	14	18	69	4	6
Adenylic-guanylic dinucleotide	83	14	18	69	4	6
Adenylic-uridylic dinucleotide	83	14	18	69	4	6
Guanylic-cytidylic dinucleotide	83	14	18	69	4	6

acid incorporation system which we have described there is, as yet, no inkling of such specificity. On the contrary, the findings in nuclei deprived of their original DNA suggest a rather generalized ability of polynucleotide fragments to facilitate amino acid incorporation. In the intact nucleus this ability is very probably linked with factors of specificity in the intact DNA. Yet the ability of other DNAs, DNA fragments, and RNA to substitute for thymus DNA in these experiments introduces some questions of considerable interest. Does specificity reside in the chromosomal protein as well as in the DNA? Evidence has already been presented to show that even the smaller proteins of the

chromosome are retained by DNAase-treated nuclei. If the proteins originally associated with DNA retain their specific configurations, then added polynucleotide may be taken up only to conform with the original spatial arrangement of the native DNA. Moreover, added polynucleotides might serve to stabilize synthetic "machinery" which would be extremely labile once the native DNA had been removed. If this is the case, then the proteins synthesized by "restored" DNAase-treated nuclei might be the same, regardless

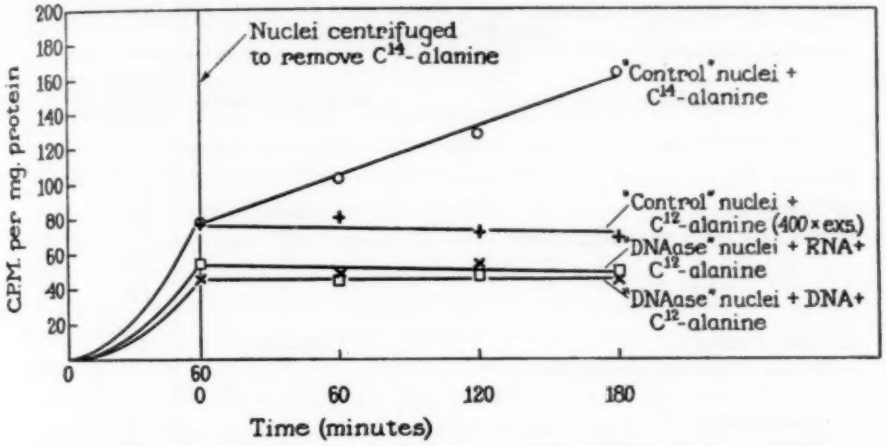

FIG. 12. The retention of the alanine-1-C^{14} incorporated into the proteins of DNAase-treated nuclei which were restored by adding thymus DNA or yeast RNA. (See text.) The nuclei were treated with DNAase, then given a nucleic acid supplement and incubated in the presence of C^{14}-alanine. They were then centrifuged down and reincubated in the presence of a great excess of unlabelled L-alanine. The specific activity of the nuclear protein at different intervals after adding the unlabelled alanine is plotted against time. The upper curve shows that the nuclei in these experiments are still able to incorporate C^{14}-amino acid.

of the source of added nucleic acid. On the other hand, if specificity resides only in the nucleic acid, then the possibility exists that added nucleic acids lead to the synthesis by the nucleus of different proteins. This would be analogous to bacteriophage DNA dominating and modifying the metabolism of the cell which it infects. This possibility that different proteins are made in the presence of different nucleic acids is being investigated. An alternate possibility, of course, is that the nucleus uses added polynucleotides for the synthesis of its own, characteristic DNA (but cannot use nucleoside 2′ or 3′ phosphates). The ability of isolated nuclei to synthesize nucleic acids from small precursors is demonstrated below.

The ability of RNA to restore alanine uptake in nuclei deprived of their

DNA naturally raises the question of the role of ribonucleic acid in nuclear metabolism. Isolated thymus nuclei contain only a small amount of ribonucleic acid. (Analysis of the RNAase digest of acid-washed nuclei, using the orcinol procedure, shows an RNA content equal to 1.4 to 2.2 per cent of the lipid-free dry weight. The more specific *p*-bromophenylhydrazine method recently introduced by Webb (37) shows an RNA content of 1.4 per cent.) At-

TABLE X

Effect of Methyl Green, Protamine, and a Histone upon C^{14}-Amino Acid Incorporation by Isolated Calf Thymus Nuclei

Conditions of experiment	Isotope administered as	Specific activity of nuclear protein	Change in C^{14} uptake
		C.P.M./mg.	*per cent*
1. Control nuclei	DL-Alanine-1-C^{14}	154	—
Nuclei + 0.125 mg. methyl green	"	167	+8
" + 0.25 " " "	"	162	+5
" + 0.50 " " "	"	116	−25
2. Control nuclei	Glycine-1-C^{14}	205	—
Nuclei + 0.25 mg. methyl green	"	251	+22
3. Control nuclei	"	89	—
Nuclei + 0.50 mg. methyl green	"	64	−28
4. Control nuclei	DL-Alanine-1-C^{14}	154	—
Nuclei + 0.375 mg. protamine	"	168	+9
" + 0.75 " "	"	159	+3
" + 1.50 " "	"	164	+6
5. Control nuclei	Glycine-1-C^{14}	79	—
Nuclei + 1.5 mg. protamine	"	70	−11
" + " " histone (lysine-rich)	"	76	−4

tempts to influence nuclear protein synthesis by removing this small amount of RNA have not so far been successful. Treatment of the nuclei with ribonuclease before adding C^{14}-amino acids has no effect on the uptake of the latter. However, this result does not permit the conclusion that ribonucleic acid has no part in nuclear protein synthesis, because in the experiments mentioned only 54 per cent of the nuclear RNA could be released by the action of the enzyme. The refractory nature of RNA-binding in the thymus nucleus is also evidenced by the fact that the DNA can be removed without appreciably influencing the RNA content. There is, however, another line of attack, directed against nuclear RNA synthesis, and evidence is presented below which suggests that the RNA of the nucleus has an important role in initiating the synthesis of some nuclear proteins.

It was previously noted that the uptake of isotopic amino acids into the proteins of thymus nuclei is not a rapidly reversible, exchange type reaction. This conclusion was obtained from experiments which showed that labelled amino acids once incorporated into nuclear proteins are not readily replaced by non-labelled amino acids in the medium. Similar experiments were performed on DNAase-treated nuclei which were supplemented with either thymus DNA or yeast RNA. These tests are summarized in Fig. 12. In both cases the uptake was apparently irreversible: the nuclei did not lose isotopic amino acid once it was incorporated. The failure to exchange cannot be the result of a loss of activity because such nuclei given C^{14}-alanine show an extensive C^{14} uptake into their proteins.

Since, in the intact nucleus, deoxyribonucleic acid is required for protein synthesis the question naturally arises as to how the DNA participates in amino acid incorporation. Several experiments were performed to test whether the action of deoxyribonucleic acid requires the active participation of its free phosphate groups. The results of these experiments are summarized in Table X. The procedure used was to add varying amounts of basic compounds which are known to combine with the free phosphoric acid groups of deoxyribonucleic acid. The compounds selected included the basic dye, methyl green, and two basic proteins, protamine, and a lysine-rich histone prepared from calf thymus. It is clear that the addition of small amounts of methyl green, protamine, or histone to the nuclei before adding the isotopic amino acid has little effect on the uptake. Larger amounts lead to a partial impairment of carbon-14 incorporation; but the impairment may reflect osmotic or other damage to the system, since large amounts of added serum albumin also decrease the uptake. The results suggest that some of the free phosphate groups of the DNA can be blocked without affecting the uptake of labelled amino acids.

Which Nuclear Proteins Become Labelled?—The answer to this question depends at the outset upon the existence of a dependable and meaningful scheme of fractionating the proteins of the nucleus. This is a field of endeavor which is now in its earliest stages of development, though it promises to become a major topic of biochemical investigation.

We have developed a provisional fractionation procedure which is applicable to the proteins of thymus sucrose nuclei, and which permits the separation of several classes of proteins of different properties and considerable biological interest. In its essentials the procedure is as follows: (See flow-sheet in Fig. 13.)

1. The nuclei are incubated in buffered sucrose solution in the presence (in these experiments) of C^{14}-alanine. After uptake has occurred the nuclei are centrifuged down. The protein released into the medium is precipitated with trichloroacetic acid (TCA). The amount of this protein in the medium increases with the time of incubation, but it has a very low specific activity

relative to the other proteins of the nucleus. The uptake into this fraction does not seem to be dependent upon DNA since pretreatment of the nuclei with DNAase does not affect it; nor does RNAase have any effect.

2. The nuclei are next extracted with 0.1 M potassium phosphate buffer at pH 7.1. The extract contains some RNA and a small amount of protein, the yield of which seems to diminish as the incubation is prolonged. This protein

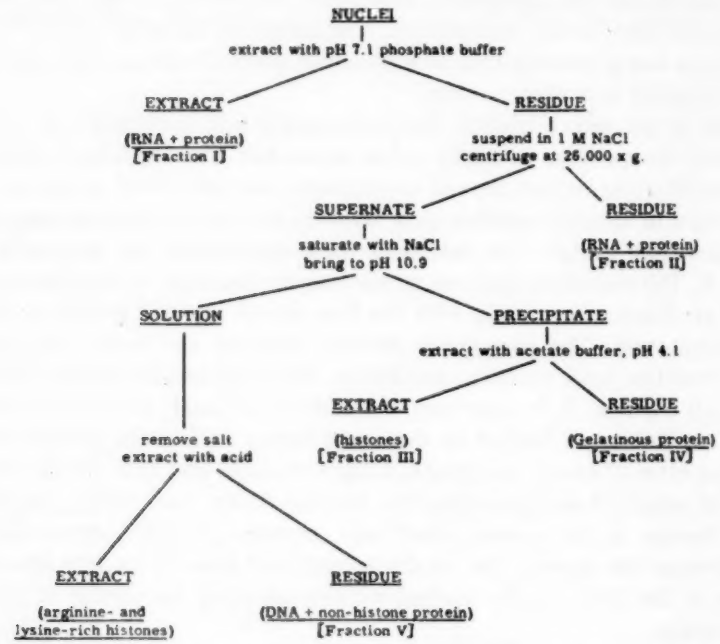

FIG. 13. Flow-sheet for the fractionation of the proteins of isolated thymus nuclei (see text).

[fraction I] has considerable interest because at times it is the most actively labelled fraction of the nucleus. Moreover, the uptake of isotope into this fraction is almost completely abolished by pretreating the nuclei with DNAase. It is not affected by pretreatment with RNAase.

3. The nuclei are then suspended in 1 M sodium chloride solution, in which they swell and largely dissolve. The opalescent solution is centrifuged at 26,000 $\times$ g to sediment the undissolved material. This residue [fraction II] comprises about 10 per cent of the nuclear mass and contains one-third of the nuclear ribonucleic acid. There is evidence to suggest that this fraction contains the nucleoli (25).

4. The supernatant after high speed centrifugation contains the bulk of the protein of the nucleus, and all the deoxyribonucleic acid. It can be frac-

tionated to yield different histones, a gelatinous protein, and an unfractionated residue. The protein in the latter is firmly attached to the DNA. (See reference (2) and experimental section.)

The distribution of C^{14} activity in these different protein fractions is a

TABLE XI

Alanine-1-C^{14} Incorporation into Different Protein Fractions of Thymus Nuclei

Protein fraction	Incubation time	Specific activity
	min.	*c.p.m./mg.*
1. Total nuclear protein	90	107
Fraction I (protein-soluble in pH 7.1 buffer)	"	179
" II (residue after extraction in 1 M NaCl)	"	117
" III (histones)	"	36
" IV (gelatinous protein)	"	157
" V (non-histone protein associated with DNA)	"	243
2. Protein released to medium during incubation	30	5
	60	8
	90	16
Fraction I	30	10
	60	66
	90	105
Arginine-rich histones	30	2.3
	60	4.5
	90	6.7
Lysine-rich histones	30	—
	60	5.6
	90	6.9
3. Control nuclei		
Protein released to medium during incubation	90	41
Fraction I	"	323
Other fractions combined	"	64
DNAase-treated nuclei		
Protein released to medium during incubation	90	47
Fraction I	"	25
Other fractions combined	"	5

matter of some interest (Table XI). (It should be mentioned that the total alanine contents of the protein fractions are very similar, so that differences in carbon-14 concentration reflect differences in the metabolic activity of the various proteins.) It was found that the protein most closely associated with the DNA is more active than all other proteins of the nucleus, with the occasional exception of the small fraction extractable in pH 7.1 buffer. This result lends further support to the conclusion drawn from deoxyribonuclease experiments that the DNA plays a role in protein synthesis. A second point

of interest is the fact that the level of isotope incorporation into the histone proteins is relatively low. This result agrees with earlier *in vivo* experiments in which it was noted that the incorporation of N^{15}-glycine into the histones of mouse liver, pancreas, and kidney is much lower than the uptake into the residual proteins of the chromosome (26).

A final point deals with the effect of pH upon amino acid incorporation into the different protein fractions of isolated nuclei. When nuclei are incubated at pH 6.7, the uptake of C^{14}-alanine into the DNA-attached protein is only about half of that observed following incubation at pH 7.3. In contrast, the uptake into the proteins of the "pH 7.1 extract" is slightly higher when the nuclei are incubated at pH 6.7.

Nucleic Acid Synthesis.—The question now arises as to whether the isolated thymus nucleus can synthesize nucleic acid by itself. This was tested by incubating the nuclei in the presence of C^{14}-labelled glycine, a substance known to be a precursor of the purine ring in nucleic acid biosynthesis. Table XII summarizes some preliminary experiments in which incorporation was demonstrated in three ways. In the first, the total nucleic acid was extracted with dilute KOH and prepared by zone electrophoresis. It may contain a trace of adsorbed protein. In the other experiments the nucleotides and the purines were separated on ion exchange resins. In all cases, a detectable amount of isotope appeared in the nucleic acid.

TABLE XII

Incorporation of Glycine-1-C^{14} into the Proteins and Nucleic Acids of Isolated Calf Thymus Nuclei

Nuclear component	Specific activity
	c.p.m./mg.
Total nuclear protein	117
Nucleic acid prepared by zone electrophoresis	19
Nucleotides prepared on dowex 1	11
Purines prepared on dowex 50	57

A further study of nucleic acid synthesis in isolated nuclei was carried out using orotic acid–6-C^{14} as precursor of the nucleic acid pyrimidines. In these experiments the uptake of radiocarbon into the *ribonucleic* acids of the nucleus was followed by incubating nuclei at 37° in the usual buffered sucrose medium in the presence of orotic acid–6-C^{14}. After washing to remove acid-soluble nucleotides, the nuclear RNA was prepared by treating the nuclei with ribonuclease: the released RNA fragments were adsorbed on charcoal for counting. We also found that some nucleic acid was readily extracted from acid-washed nuclei when they were placed in 0.2 M phosphate buffer at pH 7. This material was adsorbed and counted separately. The results of several such experiments are summarized in Table XIII. Separate columns

list the specific activity of RNA selectively released by RNAase, the nucleic acid extracted by the buffer, and the combined fractions. It is clear that a rapid and considerable uptake of orotic acid takes place, and that there is a well defined time course of C^{14} incorporation into nuclear RNA.

TABLE XIII

Orotic Acid-6-C^{14} Incorporation into Nuclear Ribonucleic Acids

Conditions of experiment	Incubation time	Specific activity of		
		RNA released by RNAase	NA soluble in buffer	Combined NA fractions
	min.	*C.P.M./mg.*	*C.P.M./mg.*	*C.P.M./mg.*
1. Control nuclei*	30	85	202	147
	60	142	306	220
	90	385	420	402
	120	443	472	457
Nuclei + 0.1 mg. DRB at t = 0 min.	30	0	136	62
	60	0	191	87
	90	93	234	161
	120	180	237	206
Nuclei + 0.1 mg. DRB at t = 30 min.	30	89	202	144
	60	93	—	152
	90	93	287	176
	120	54	349	179
2. Control nuclei‡	30	290	1270	567
	60	625	1640	938
	90	690	1750	1030
Nuclei + 0.1 mg. DRB at t = 0 min.	30	91	715	253
	60	100	925	288
	90	225	833	427
Nuclei + 0.1 mg. DRB at t = 30 min.	30	364	1270	641
	60	397	1105	617
	90	339	873	500

* Each flask (containing 30 mg. of nuclei in 2.0 ml. of incubation medium) received 0.075 mg. orotic acid-6-C^{14}; specific activity 1.0 mc./mM.

‡ Each flask received 0.20 mg. orotic acid-6-C^{14}.

The synthesis of ribonucleic acid by the nucleus seems to require the presence of deoxyribonucleic acid. In preliminary experiments it was found that pretreatment of the nuclei with DNAase greatly reduced subsequent orotic acid incorporation into the RNA. For example, in nuclei which had lost 68 per cent of their DNA, the ribonucleic acid had a specific activity of only 21 C.P.M. per mg. after 1 hour's incubation. The corresponding figure for un-

treated controls was 186 C.P.M. per mg. of RNA. The effect of a DNA supplement on nucleic acid synthesis by DNAase-treated nuclei has not yet been tested. (In a recent communication, Friedkin and Wood report the incorporation of C^{14}-thymidine into the DNA of isolated rabbit thymus nuclei. In their experiments, too, the presence of DNAase lowers the incorporation (36).)

It was previously pointed out that 5,6-dichloro-β-D-ribofuranosyl-benzimidazole (DRB) is an inhibitor of protein synthesis in isolated thymus nuclei, and that one of the most striking properties of this inhibitor is the time limit of its effectiveness. DRB is an effective inhibitor of nuclear protein synthesis only if it is added at the outset of the incubation. If alanine-C^{14} uptake is allowed to proceed before adding the DRB, little or no subsequent inhibition is observed. These results suggest that in the first 30 minutes some activation or "priming" process takes place within the nucleus which permits protein synthesis. Once the activation has occurred, DRB cannot inhibit further amino acid uptake.

Now what is the nature of the "DRB-sensitive" activation process? The structure of DRB suggests that it may function as a purine nucleoside antagonist and interfere with the synthesis of nucleic acid. To test this hypothesis we studied the effect of DRB on RNA synthesis by isolated nuclei. The results are included in Table XIII. In control nuclei one observes a straightforward time course of orotic acid-C^{14} incorporation into nuclear RNA. But when DRB is added to the medium at the outset of the experiment, C^{14} uptake is greatly reduced. The addition of DRB 30 minutes after the beginning of the experiment also effectively inhibits further RNA synthesis. Thus DRB seems to block the synthesis of ribonucleic acid within the nucleus no matter when it is added to the incubation medium. On the other hand, DRB inhibits nuclear protein synthesis only when it is present from the beginning.

These results suggest that the activation or priming of the nucleus so that it actively incorporates amino acids into its proteins requires a preliminary synthesis of ribonucleic acid. Once this nucleic acid has been laid down, protein synthesis begins. Subsequent interference with RNA synthesis does not appreciably affect the level of amino acid uptake (in the time interval studied in these experiments). The question now arises as to whether the "activation" of the nucleus involves the synthesis of an autonomous system, which then takes over protein synthesis independently of the DNA. This was tested in experiments in which the DNA was not removed until activation had occurred. It was found that removal of the DNA after a preliminary 30 minute incubation stopped protein synthesis just as effectively as it always does in unincubated samples. It follows that the requirement for DNA in nuclear protein synthesis remains even after activation has occurred. Our studies have shown that the DNA of the nucleus is essential for both RNA and pro-

tein synthesis. It remains to be seen whether DNA *synthesis* is required for any of these essential biosynthetic reactions.

EXPERIMENTAL

Preparation of Nuclei.—The procedure used is a modification of that described by Schneider and Petermann (29). All operations were carried out at 2°C. 50 grams of fresh calf thymus tissue was finely minced with scissors and placed in a blendor vessel together with 50 ml. of 0.5 M sucrose solution and 400 ml. of 0.25 M sucrose–0.0033 M $CaCl_2$. (This calcium concentration is higher than that previously described (1) because larger amounts of Ca^{++} ion prevent the gel formation occasionally obtained in the earlier procedure.) The tissue was gently homogenized by running the blendor at 1000 R.P.M. for 4 minutes. The resulting homogenate was filtered through a double layer of gauze (Johnson and Johnson type I) and then through a single thickness of double napped flannelette. The filtrate was centrifuged at 2400 R.P.M. (1000 × *g*) for 7 minutes and the supernate was discarded. The sediment was resuspended in 100 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$ solution and the suspension was again passed through flannelette. The filtrate was centrifuged at 1000 × g for 7 minutes. The sedimented nuclei were again washed in the centrifuge using 100 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$. Further washings were rarely necessary, since the supernate by this time was usually water-clear. The nuclei were finally suspended in 40 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$, a dilution which usually provided 30 to 50 mg. of nuclei (dry weight) per ml. of suspension.

Incubation Procedure.—Aliquots of the nuclear suspension were incubated aerobically at 37° in the presence of isotopically labelled amino acid, buffer, and added metabolites, as follows: each incubation vessel contained (1) 1.0 ml. of nuclear suspension, (2) 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer, (3) 0.4 ml. of 0.1 M glucose solution containing 3.75 mg. NaCl per ml., and (4) 0.1 ml. of water containing 0.05 mg. of radioactive amino acid. The amino acids used were DL-alanine-1-C^{14} (specific activity 1.4 mc./mM), glycine-1-C^{14} (specific activity 1.35 mc./mM), DL-lysine-2-C^{14} (specific activity 0.33 mc./mM), and L-methionine-S^{35} (specific activity 4.4 mc./mM). Most of the experiments described were carried out at pH 6.7 or pH 7.3. Uptakes are higher at the higher pH but under these conditions nuclei formed a heavy gel which was difficult to centrifuge and which precluded a clean separation of nuclei from suspending medium. When such separations were necessary, as in the DNAase experiments and tests for amino acid exchange, the pH was held at 6.7 and occasionally at 6.3. The flasks were immersed in a 37° water bath and shaken at 140 cycles per minute for the required period, usually 1 hour. In experiments in which the proteins of the nucleus were to be subsequently fractionated, the uptake was halted by the addition of 0.2 ml. of 0.1 M NaCN solution. In all other cases the reaction was stopped by the addition of an equal volume (2.0 ml.) of 20 per cent trichloroacetic acid.

In testing the effects of various inhibitors (Tables I and III) these substances were dissolved in the phosphate-sucrose buffer at four times the final concentrations listed in the table.

In comparing glucose, fructose, and α-ketoglutarate (Table II) these substances

were prepared as 0.1 M solutions containing the required amount of sodium: 0.4 ml. of each solution was used, together with 1.0 ml. of nuclear suspension, 0.5 ml. of buffer, and 0.1 ml. of the amino acid solution.

In testing the effects of the substituted benzimidazoles (Table IV and Fig. 5), the compounds were dissolved in 0.25 M sucrose–0.0030 M $CaCl_2$. One-tenth ml. of solution containing the indicated amounts of inhibitor was added to the incubation mixture at 0, 30, or 60 minutes. Controls received 0.1 ml. of sucrose–$CaCl_2$. The time course of incorporation in control nuclei and in nuclei receiving the inhibitor was followed by halting the uptake of different flasks at 30 minute intervals.

In studying the effect of varying sodium concentration upon amino acid uptake (Fig. 6), the incubation mixture contained 1.0 ml. of nuclear suspension, 0.5 ml. of 0.03 M potassium phosphate–sucrose buffer at pH 7.3, 0.4 ml. of 0.1 M glucose containing varied amounts of sodium chloride, and 0.1 ml. of glycine-1-C^{14} solution containing 1.0 mg. = 0.018 mc./ml. Uptake was allowed to proceed for 1 hour.

The change in amino acid incorporation with changing sodium/potassium ratios (Fig. 7) was measured in incubation mixtures containing 1.0 ml. of nuclear suspension, 0.5 ml. of 0.03 M potassium phosphate–0.175 M sucrose buffer at pH 7.3, 0.4 ml. of 0.1 M glucose containing 0.137 mM of total chlorides, the ratio of sodium to potassium being varied as indicated in the chart, and 0.1 ml. of glycine-1-C^{14} solution containing 1.0 mg. = 0.018 mc./ml.

The effect of sucrose concentration upon alanine-C^{14} uptake (Fig. 8) was studied using nuclei which had been isolated and suspended in 0.25 M sucrose–0.0030 M $CaCl_2$. The incubation mixture contained 1.0 ml. of nuclear suspension, 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl per ml., 0.1 ml. of DL-alanine-1-C^{14} solution containing 0.5 mg. = 0.008 mc./ml., and 0.5 ml. of 0.1 M sodium phosphate buffer (pH 6.7) containing varying amounts of sucrose (between 0 and 1.5 M) to give the final concentrations plotted in the chart. Uptake was stopped after 60 minutes.

In testing whether nuclei utilize the D-form of administered amino acids (Fig. 9) the incubation mixture comprised 1.0 ml. of nuclear suspension, 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 7.3) containing varied amounts of either unlabelled D-alanine or unlabelled L-alanine, as indicated in the chart, 0.4 ml. of 0.1 M glucose containing 6.25 mg. NaCl per ml., and 0.1 ml. of DL-alanine-1-C^{14} solution containing 0.5 mg. = 0.008 mc./ml. Incorporation was allowed to proceed for 60 minutes.

The test for exchange of amino acid once incorporated (Fig. 10) was carried out as follows: nuclei were incubated in the usual way (but at pH 6.3 to minimize gel formation) for 60 minutes in the presence of 0.05 mg. of DL-alanine-1-C^{14}. At that time the suspensions were transferred to centrifuge tubes (12 ml. capacity, with conical tip) and the nuclei were sedimented at 2600 R.P.M. for 5 minutes. The supernate was removed and the nuclei were resuspended in 1.0 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$, 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 6.3), and 0.4 ml. of 0.1 M glucose containing 7.5 mg. NaCl per ml. Control flasks received 0.1 ml. H_2O. Other flasks received 0.1 ml. of the DL-alanine-1-C^{14} solution used previously (to test whether nuclei after centrifugation and resuspension could still incorporate amino acid). The experimental flasks received 0.1 ml. of unlabelled L-alanine solution containing 5 mg. alanine/ml. This supplied more than 200 times as much unlabelled

amino acid as C^{14}-alanine remaining. The nuclei were then incubated at 37°. The reaction was followed by halting the uptake of different flasks at 30 minute intervals, and the protein was prepared as described below. Other controls using unlabelled D-alanine, showed that the amount of unlabelled amino acid added did not lead to osmotic or other damage to the system.

Similar experiments, using S^{35}-labelled methionine, were carried out by the same general procedure. These experiments were performed at pH 6.7. The conclusions are essentially the same as those derived from C^{14}-alanine experiments.

In studying the effect of removing DNA on the incorporation of alanine-1-C^{14} and lysine-2-C^{14} (Fig. 11), the following procedure was used: each flask contained 1.0 ml. of nuclear suspension and 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl and 4.19 mg. $MgCl_2 \cdot 4H_2O$ per ml. Control flasks received 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer at pH 6.7. Other flasks received this buffer containing increasing amounts of crystalline pancreatic deoxyribonuclease (Worthington). Enzyme concentrations in the buffer ranged from 0.125 to 4.0 mg. per ml. The suspensions were incubated at 37° for 30 minutes, and the nuclei were centrifuged down at 2600 R.P.M. for 5 minutes. The supernates were carefully removed and the amount of nucleic acid which they contained was measured spectrophotometrically at 260 mμ. The nuclei were then resuspended in 1.0 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$ containing 2.94 mg. Na_3 citrate $\cdot 2H_2O$ per ml., 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 6.7), 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl per ml., and 0.1 ml. of H_2O containing 0.05 mg. of either DL-alanine-1-C^{14} or DL-lysine-2-C^{14}. Incorporation was allowed to proceed for 60 minutes, when it was halted by the addition of an equal volume of 20 per cent trichloroacetic acid (TCA).

The "restoration" of amino acid incorporation in DNAase-treated nuclei (Tables V, VII, VIII, and IX) was obtained as follows: each flask contained 1.0 ml. of nuclear suspension and 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl and 4.19 mg. $MgCl_2 \cdot 4H_2O$ per ml. Control flasks received 0.5 ml. of the phosphate-sucrose buffer at pH 6.7. Other flasks received this buffer containing 2.0 mg. deoxyribonuclease per ml. The suspensions were incubated at 37° for 30 minutes, and the nuclei centrifuged down as described above. The amount of nucleic acid released into the supernate was measured by the E_{260}. The nuclei were then resuspended in 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer, 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl per ml., and 0.1 ml. of isotopic amino acid solution. Some of the flasks received 1.0 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$ containing 2.94 mg. Na_3 citrate $\cdot 2\ H_2O$ per ml. and nothing else. Other flasks (containing DNAase-treated or control nuclei) received 1.0 ml. of the sucrose-citrate solution containing 5 mg. of DNA (or other supplement) per ml. The flasks were then incubated at 37° for 60 minutes and the uptake was stopped by the addition of an equal volume of 20 per cent TCA. The C^{14} incorporations into the proteins of the control nuclei were compared with those observed in DNAase-treated nuclei, and in treated nuclei which had received a nucleic acid supplement. The supplements used had little or no effect on amino acid uptake by control nuclei.

A number of other controls were run in these experiments. In some cases DNA was added to DNAase-treated nuclei after the incubation with labelled amino acids. The purpose of this experiment was to see whether an actively labelled fraction exists

which might be formed by DNAase-treated nuclei and be coprecipitated only in the presence of added DNA. (Such a fraction, if it existed, would have to be soluble in 10 per cent TCA.) No evidence for such an elusive, active protein was found. DNA added after incubation with C^{14}-alanine did not lead to higher specific activities of the nuclear protein. In other experiments, nuclei were allowed to incorporate C^{14}-alanine before treatment with DNAase. They were subsequently treated with the enzyme, and an active protein fraction was searched for in the medium. These experiments showed that DNAase- treated nuclei lost none of their counts: all the activity remained in TCA-precipitable nuclear protein.

The nucleic acids used were prepared by methods previously described (30, 31). The apurinic acid was prepared by the method of Tamm, Hodes, and Chargaff (32).

The test for exchange of amino acids taken up by restored DNAase-treated nuclei (Fig. 12) was carried out by combining two of the above procedures. Nuclei were treated with DNAase, centrifuged, and resuspended in the presence of thymus DNA or yeast RNA and C^{14}-alanine, as described above. After 60 minutes' incubation, the nuclei were centrifuged down, and the supernate was discarded. The nuclei were then resuspended in 1.0 ml. of 0.25 M sucrose–0.0030 M $CaCl_2$, 0.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 6.7), and 0.4 ml. of 0.1 M glucose containing 3.75 mg. NaCl per ml. Control flasks received 0.1 ml. H_2O. Other flasks received 0.1 ml. H_2O containing 0.05 mg. DL-alanine-1-C^{14} (to test whether these nuclei could still incorporate amino acid). The experimental flasks received 0.1 ml. of unlabelled L-alanine solution (10 mg./ml.). This supplied more than 400 times as much unlabelled amino acid as C^{14}-alanine remaining. The nuclei were then incubated at 37°. The reaction was followed by halting the uptake of different flasks at 30 minute intervals. The protein was prepared as described below.

In testing the effects of methyl green and basic proteins upon amino acid incorporation (Table X), these substances were dissolved in the phosphate-sucrose buffer so that 0.5 ml. contained the amounts listed in the table. The methyl green was recrystallized as the perchlorate. The lysine-rich histone was prepared by procedures previously described (15). The protamine was a preparation of the Lilly Research Laboratories. Uptake was halted after 60 minutes by the addition of an equal volume of 10 per cent TCA.

Protein Fractionation.—For measuring alanine-1-C^{14} incorporation into different protein fractions of the nucleus (Fig. 13 and Experiment 1 in Table XI) the scale of the experiments was increased by a factor of 25. To 25 ml. of nuclear suspension was added 12.5 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 7.3), 10 ml. of 0.1 M glucose containing 6.25 mg. NaCl per ml., and 2.5 ml. H_2O containing 1.25 mg. DL-alanine-1-C^{14}. The suspension was shaken at 37° for 90 minutes. It was then cooled in an ice bath and fractionated as follows:—

1. The nuclear gel was centrifuged down, and a small portion of the sediment taken for a measure of C^{14} uptake into the total nuclear protein.

2. All subsequent operations were carried out at 2°C. The residue was minced with scissors and extracted with 50 ml. of 0.1 M potassium phosphate buffer at pH 7.1 for 10 minutes. (In later experiments this extraction was carried out by homogenizing the nuclear gel in the blendor for 30 seconds.) The suspension was centrifuged and the clear supernate was carefully decanted. This pH 7.1 extract (fraction I) was

precipitated by adding TCA to a final concentration of 10 per cent. The proteins were prepared for counting as described below.

3. To the nuclear residue an equal volume of 2 M NaCl was added. The gel was stirred in a high-speed mixer for 3 hours. It was then centrifuged at 26,000 R.P.M. in the Spinco ultracentrifuge for 2 hours. The sediment (fraction II) was washed with 1 M NaCl and prepared for counting as described below.

4. The supernate was saturated with NaCl and brought to pH 10.9 by the careful addition of 1 N NaOH. After stirring for 20 minutes, the suspension was centrifuged to remove the precipitated histones and gelatinous protein. The supernate (A) was set aside. The sediment was washed with saturated NaCl solution at pH 10.9. It was then taken up in water (about 15 ml.) and dialyzed overnight. The suspension was centrifuged, and the supernate (which contains the histones) was set aside. The gelatinous residue was extracted with 8 ml. of 0.1 M acetate buffer at pH 4.1 and recentrifuged at 12000 R.P.M. This extraction was repeated, and both extracts were added to the original supernate containing the histones. The residue of gelatinous protein (fraction IV) was prepared for counting as described below.

5. The combined supernates were brought to pH 11 with NaOH. The precipitated histones (fraction III) were washed with ethanol and prepared for counting.

6. The proteins of supernate (A) were next fractionated by adding 2 N HCl to bring the final acid concentration to 0.2 N. The DNA-protein precipitate was centrifuged down. The residue was reextracted with 0.2 N HCl, and the precipitate (fraction V) prepared for counting as described below.

7. The supernate contains both arginine-rich and lysine-rich histones, which can be fractionated by methods previously described (15).

Preparation of Protein for Counting.—In most experiments the over-all specific activity of the total nuclear protein was determined. The procedure was to halt incorporation by the addition of an equal volume of 20 per cent TCA. The suspension was brought to a volume of 10 ml. with 10 per cent TCA and centrifuged (2600 R.P.M. for 10 minutes). The supernate was discarded. The residue was washed three times in the centrifuge with 10 ml. portions of 10 per cent TCA. The washings were discarded. The residue was taken up in 10 ml. of 10 per cent TCA, brought to 90°C. for 20 minutes, and centrifuged. The supernate, which contains the nucleic acids, was discarded. The residue was again washed with 10 per cent TCA. To remove lipides the precipitate was washed once with hot 95 per cent ethanol, twice with a warm 2:2:1 ethanol-ether-chloroform mixture, and once with ether. The protein was then air-dried at room temperature. The dry protein was resuspended in acetone, transferred to a thick-walled glass tube, and homogenized using a motor-driven Teflon pestle. The protein dispersion was deposited on filter paper (Whatman No. 50) using the Tracerlab E8A filtration apparatus. The activity of the samples was counted using a thin window Geiger-Müller tube and scaling circuit. Self-absorption corrections were made by the equational method of Schweitzer and Stein (6), using an experimentally determined value of b equal to 7.0.

Nucleic Acid Synthesis.—In measuring glycine-1-C^{14} uptake into nucleic acid purines (Table XII) three procedures were used. In all cases the incubation mixture consisted of 6.0 ml. of nuclear suspension, 3 ml. of 0.1 M sodium phosphate–0.25 M sucrose buffer (pH 7.3), 2.4 ml. of 0.1 M glucose containing 6.25 mg. NaCl per ml.,

and 0.6 ml. of H_2O containing 0.6 mg. glycine-1-C^{14}. The flasks were shaken at 37° for 60 minutes, at which time uptake was stopped by adding an equal volume of 10 per cent TCA. The suspensions were centrifuged, and the nuclei were washed once with cold 5 per cent TCA and once with cold 2 per cent perchloric acid. Lipides were then removed by washing once with hot ethanol, and once with warm 2:2:1 ethanol-ether-chloroform mixture.

1. In the first procedure, the total nucleic acid was solubilized by treating the residue in 1 N KOH for 16 hours. The suspension was then brought to pH 7.8, (using 20 per cent $HClO_4$), cooled, and centrifuged. The supernate was placed on a starch block and subjected to electrophoresis, following the procedure of Kunkel and Slater (33). The block was then cut into segments which were extracted with water. The location and amount of the nucleic acid were easily determined by the E_{260}. The eluates containing nucleic acid were lyophilized. The lyophilized material was redissolved and again subjected to electrophoresis. The nucleic acids were eluted with water and lyophilized. The dry powder was homogenized in acetone and plated for counting.

2. In a second procedure, the nucleotides were separated on an ion exchange resin. The acid-washed, lipid-free nuclei were extracted with 30 ml. of 10 per cent $HClO_4$ at 70°C. for 20 minutes. The pH was adjusted to 6.7 with 6 N KOH and the suspension was centrifuged in the cold. The supernate was passed through a column of dowex 1–formate (34), and the column was washed with water. The nucleotides were eluted with 4 N formic acid containing 1 M ammonium formate. The eluate was then lyophilized. The radioactivity of the nucleotides was determined in the usual way.

3. In the third procedure, the purines were separated on a dowex-50 column. The acid-washed, lipid-free nuclei were treated with 20 ml. of 5 per cent TCA at 90° for 15 minutes and centrifuged. The supernate was repeatedly shaken with ether to remove the TCA, and concentrated HCl was added to bring the final concentration to 1 N. The solution was placed in a boiling water bath for 1 hour, cooled, and the acid concentration brought to 2 N with HCl. The bases were then placed on dowex 50 (H^+ form) and eluted following the method described by Cohn (35). The eluates were lyophilized and the specific activity of the mixed purines determined in the usual way.

SUMMARY

1. Nuclei prepared from calf thymus tissue in a sucrose medium actively incorporate labelled amino acids into their proteins. This is an aerobic process which is dependent on nuclear oxidative phosphorylation.

2. Evidence is presented to show that the uptake of amino acids represents nuclear protein synthesis.

3. The deoxyribonucleic acid of the nucleus plays a role in amino acid incorporation. Protein synthesis virtually ceases when the DNA is removed from the nucleus, and uptake resumes when the DNA is restored.

4. In the essential mechanism of amino acid incorporation, the role of the DNA can be filled by denatured or partially degraded DNA, by DNAs from

other tissues, and even by RNA. Purine and pyrimidine bases, monoribonucleotides, and certain dinucleotides are unable to substitute for DNA in this system.

5. When the proteins of the nucleus are fractionated and classified according to their specific activities, one finds the histones to be relatively inert. The protein fraction most closely associated with the DNA has a very high activity. A readily extractable ribonucleoprotein complex is also extremely active, and it is tempting to speculate that this may be an intermediary in nucleocytoplasmic interaction.

6. The isolated nucleus can incorporate glycine into nucleic acid purines, and orotic acid into the pyrimidines of its RNA. Orotic acid uptake into nuclear RNA requires the presence of the DNA.

7. The synthesis of ribonucleic acid can be inhibited at any time by a benzimidazole riboside (DRB) (which also retards influenza virus multiplication (11)).

8. The incorporation of amino acids into nuclear proteins seems to require a preliminary activation of the nucleus. This can be inhibited by the same benzimidazole derivative (DRB) which interferes with RNA synthesis, provided that the inhibitor is present at the outset of the incubation. DRB added 30 minutes later has no effect on nuclear protein synthesis. These results suggest that the activation of the nucleus so that it actively incorporates amino acids into its proteins requires a preliminary synthesis of ribonucleic acid.

9. Together with earlier observations (27, 28) on the incorporation of amino acids by cytoplasmic particulates, these results show that protein synthesis can occur in both nucleus and cytoplasm.

It is a pleasure to acknowledge our indebtedness to Mr. Rudolf Meudt and to Mr. Jerome Reid for technical assistance throughout the investigation.

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MONONUCLEOTIDES OF THE CELL NUCLEUS

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(Received for publication, July 18, 1956)

It has been shown in the preceding paper (1) that protein synthesis occurs within the cell nucleus. The question that then naturally arises is how the nucleus meets the energy requirement for the synthetic process. Since it has been generally accepted that in the cytoplasm energy-rich compounds such as ATP and related nucleotides participate in numerous synthetic processes, one might suppose that such compounds are involved in protein synthesis within the nucleus. It has, in fact, also been shown in the preceding paper (1) that protein synthesis in the nucleus is an oxidative process and is blocked by certain "uncoupling" reagents which are known to inhibit the synthesis of ATP in mitochondria. We have therefore investigated the nucleotide system of the nucleus and this will be described in the present paper.

Our first step has been to show that quantitatively and qualitatively much the same complement of acid-soluble mononucleotides occurs in the nucleus as in the cytoplasm. Then it has been found that within the nucleus these nucleotides can be phosphorylated to the energy-rich triphosphate form. As to the nature of the phosphorylation, there exist certain resemblances as well as differences between the nucleus and mitochondria. Another aspect of the problem that has been investigated concerns the way in which nucleotides are held in the nucleus. Finally, attempts have also been made to elucidate the role of the nuclear mononucleotides in the synthesis of protein as it occurs in the nucleus.

The work which will be mentioned below has been done on calf thymus nuclei isolated in sucrose media, unless otherwise specified. A preliminary account of this work has been published (2).

Presence of Nucleotides in the Nucleus.—When one investigates water-soluble substances such as mononucleotides in isolated nuclei, it is important to use preparations isolated in such a way that loss or adsorption of these substances is prevented during the course of nuclear isolation. Nuclei isolated in non-aqueous media (so called Behrens' type nuclei (3)) meet these requirements. Analyses of nucleotides were therefore first performed on isolated "non-aqueous" nuclei of calf thymus, and compared with the whole tissue treated

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with the same media to which the nuclei were exposed during their isolation. Cold 2 per cent perchloric acid extracts of non-aqueous nuclei and tissue were fractionated by dowex 1 ion exchange chromatography as described by Cohn and modified by Hurlbert *et al.* (4). A typical chromatogram is illustrated in

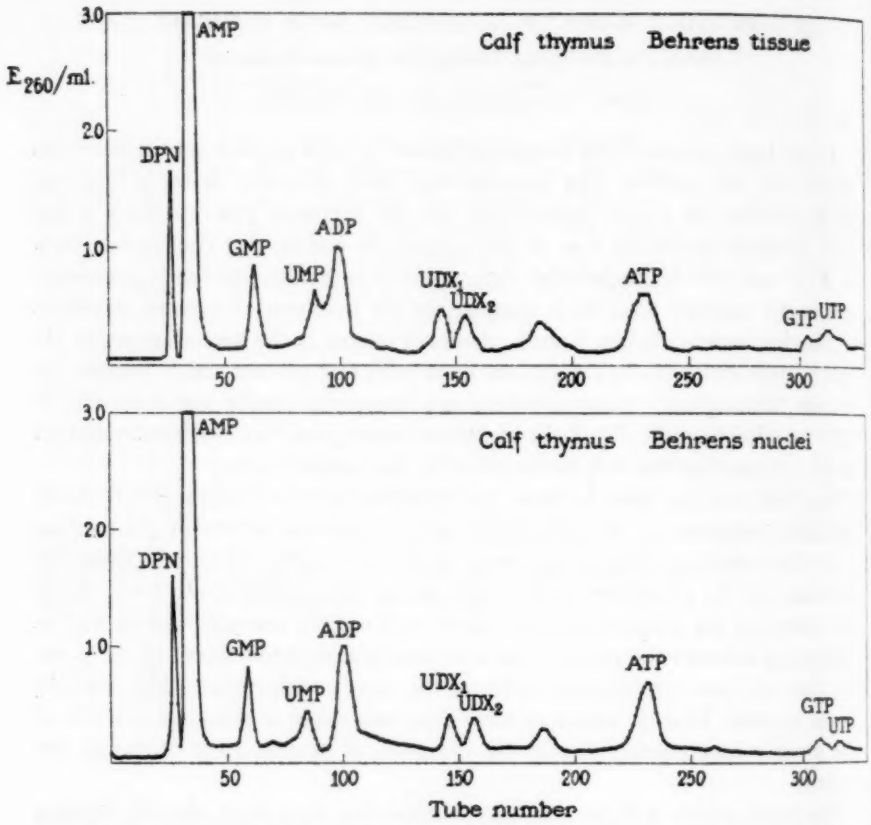

FIG. 1. Acid-soluble nucleotides of calf thymus non-aqueous nuclei and the corresponding whole tissue (see also the top column of Table I). Note the predominance of monophosphates over di- and triphosphates.

Fig. 1. It is seen that calf thymus nuclei contain quantitatively and qualitatively nearly the same complement of nucleotides as the whole tissue. Mononucleotides of adenine, guanine, cytosine, and uracil, together with several uridine diphosphate derivatives and diphosphopyridine nucleotide were identified; one of the striking features here is the predominating amount of adenine nucleotides. Only a trace of cytidine nucleotides was found. In a 2 per cent perchloric acid extract more than 80 per cent of ultraviolet-absorbing material

at 260 mμ is found to consist of nucleotides either by adding up all nucleotide peaks on the chromatogram, or by measuring at 260 mμ the quantity of light-absorbing material which did not combine with dowex 1 resin. The quantity of nucleotides in non-aqueous thymus nuclei is about 1.7 per cent of the total nuclear mass. Practically the same content of nucleotides in the nucleus as in

TABLE I

Acid-Soluble Adenine Nucleotides in Tissues and Nuclei

Material analyzed	E_{260} per gm. lipide-free dry matter present as				
	Total E_{260}	AMP	ADP	ATP	Total adenine nucleotides
Calf thymus tissue* (Behrens' preparation)	500	102	56	43	201
Calf thymus Behrens' nuclei*	500	93	58	44	195
Calf thymus Behrens' nuclei	420	80	19	14	113
Calf thymus tissue	580	119	29	15	163
Calf thymus tissue	580	118	33	20	171
Calf thymus sucrose nuclei	306	28	23	46	97
Calf thymus sucrose nuclei	390	34	22	58	104
Calf thymus sucrose nuclei	460	39	29	89	157
Calf liver tissue (Behrens' preparation)	382	—	—	—	—
Calf liver Behrens' nuclei	350	—	—	—	—
Chicken erythrocytes (Behrens' preparation)	92.0	3.2	9.3	15.8	28.3
Chicken erythrocyte (Behrens' nuclei)	95.4	6.1	14.0	14.1	34.2
Brown trout sperm	170	—	—	—	—
Rainbow trout sperm	171	—	—	—	—

* Derived from the same thymus gland.

the cytoplasm was also found in the case of calf liver and of chicken erythrocytes, indicating the general occurrence of the nucleotide system in cell nuclei. The over-all concentration of nucleotides in the nucleus is, however, different from one type of tissue to another. Nuclei from metabolically active tissues such as calf thymus, or calf liver have a higher content of nucleotides than is found in nuclei of such metabolically inert cells as chicken erythrocytes or trout spermatozoa (Table I).

When analyses of nucleotides are performed on calf thymus nuclei isolated

in 0.25 M sucrose–0.002 to 0.003 M $CaCl_2$, it is found that they usually retain 60 to 80 per cent of their original acid-soluble nucleotide content. However, the ability to retain the nucleotides in sucrose nuclei from different calf thymus tissues at different times of the year varies considerably. Such nucleotides as are retained after isolation of nuclei remain in the nuclei even after repeated washings with cold sucrose–$CaCl_2$ solution (Table II). This is rather surprising

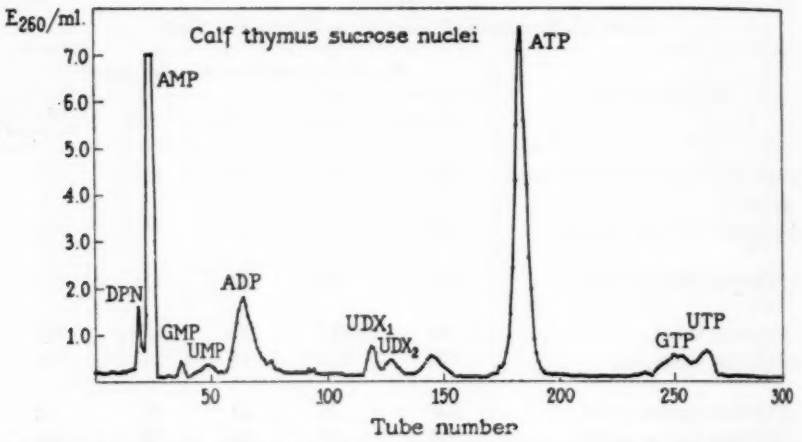

FIG. 2. Acid-soluble nucleotides of calf thymus sucrose nuclei. Note the predominance of triphosphates over di- and monophosphates.

TABLE II

Effect of Sucrose Washing on Nuclear Acid Soluble Nucleotides in Calf Thymus Sucrose Nuclei

	E_{260} per gm. lipide-free dry matter		
	Experiment 1	Experiment 2	Experiment 3
Original sucrose nuclear preparation	264	198	100
After 1st washing	234	177	90
After 2nd washing	232	—	—

because of the diffusible nature of nucleotides in aqueous media, and because the nuclear membrane of nuclei isolated in a sucrose solution is not a barrier to the passage of even much larger molecules. The way in which nucleotides are retained in the nucleus will be discussed later in this paper. Chromatographic analyses (Fig. 2, Table I) revealed that sucrose nuclei have a pattern of nucleotide distribution similar to that found in non-aqueous nuclei, with an important exception which will be dealt with in the next section.

Even before the analyses of nucleotides on isolated nuclei that have just been described were made, there were observations suggesting the presence of

these substances in nuclei: Davies (5), and Walker and Yates (6) in the course of their microspectrophotometric observations on nuclei in tissue culture, reported that there are substances which absorb ultraviolet light and are soluble in acid-alcohol fixatives. The English authors supposed that their "lower nucleotides" are precursors of DNA. However, their procedure does not permit identification of each nucleotide. Naora and Takeda (7) recognized the occurrence of labile phosphate compounds, which they assumed to be ATP, in the rat liver nuclei isolated by the Behrens' procedure. Although possible contamination of glucose-1-phosphate which is also acid-labile (8) and is known to occur in the nuclei should be considered, at least a part of their "labile phosphate" must be ATP. Edmonds and LePage (9) reported the presence of various nucleotides in the sucrose nuclei obtained from Flexner-Jobling tumor cells. Kay and Davidson (10) noted the occurrence of acid-soluble nucleotides in non-aqueous nuclei from various tissues. Now their chemical nature has been established and it is likely that nuclear mononucleotides, as cytoplasmic ones, play a part in numerous processes, including the synthesis of protein that occurs in the nucleus.

Phosphorylation of Nucleotides in the Nucleus.—The predominance of nucleotide monophosphates in thymus tissue and in thymus nuclei prepared by the non-aqueous procedure should not be taken to indicate the actual condition *in vivo*, or an artifact due to the action of non-aqueous media. We have repeatedly observed that socalled "fresh" calf thymus tissue has a similar predominance of monophosphates over di- and triphosphates (Table I). It seemed probable to us that the predominance of these low energy forms is simply a reflection of anoxia resulting from the death of the animal. Evidence to support this conclusion is derived from a series of nucleotide analyses of rat thymus tissues which can be excised from the animal after death with a speed that cannot be achieved in the case of a slaughter-house animal such as the calf. Some of the rat tissues were frozen in liquid nitrogen immediately upon excision; others were incubated at 30°C. for 5, 10, and 20 minutes. As can be seen in Fig. 3, in the immediately frozen tissue ATP predominates over ADP and AMP, and during the course of incubation the amount of ATP sharply drops with a simultaneous increase of AMP, ADP content remaining relatively unchanged. The same sort of changes were observed in the uridine and guanine nucleotides. A comparison of nucleotide distribution in non-aqueous nuclei and sucrose nuclei reveals an interesting difference between them: the predominance of low energy forms in non-aqueous nuclei and of high energy forms in sucrose nuclei. This is taken to indicate that during the preparation of sucrose nuclei in the cold, the monophosphate characteristic of the excised tissue is largely converted back to the energy-rich triphosphate form which predominates in the living tissue. The post-mortem predominance of nucleotide monophosphates in thymus tissue seems

therefore to afford a unique opportunity to study the mechanism of their phosphorylation in the nucleus.

The formation of high energy phosphate bonds is presumably the result of extensive aeration of the homogenates and nuclear suspensions during the isolation procedure. To determine whether this phosphorylation involves participation of the cytoplasmic fraction in the homogenate, or whether it is an intranuclear phenomenon, two types of experiments were carried out.

1. Nuclei were isolated as quickly as possible (at 2°C. in 25 to 28 minutes). At this time most nucleotides are still present in the monophosphate form.

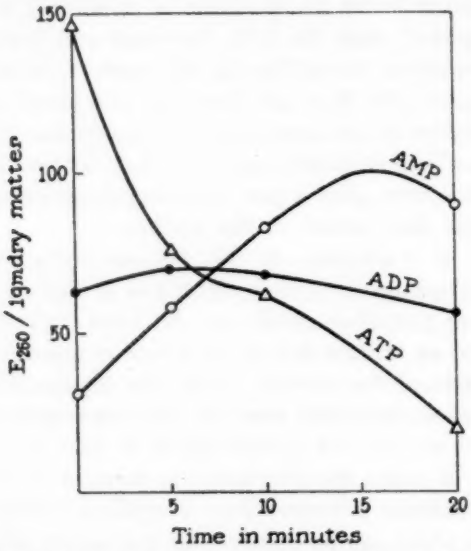

FIG. 3. Postmortem changes of adenine nucleotides in rat thymus tissue. Excised thymus tissue was incubated at 30°C. for 5, 10, 15, and 20 minutes.

Such a nuclear preparation was then gently stirred for an additional 50 to 60 minutes in the cold. At the end of this time a large part of the monophosphates had been converted into triphosphates (Table III, Fig. 4).

2. A thymus tissue homogenate in sucrose–$CaCl_2$ solution was stirred gently for 95 minutes at 4°C. This homogenate was then fractionated into nuclear and cytoplasmic components. As indicated in Table IV, the original homogenate at the beginning of the experiment contained a large amount of AMP but almost no ATP. It is obvious that during the incubation of the homogenate in the cold under the prevailing experimental conditions, phosphorylation of AMP took place in the nuclear but not in the cytoplasmic fraction, and the same may be said of the guanine and uridine nucleotides. The decrease of AMP content in the cytoplasmic fraction was probably the result of phos-

phatase action. This phosphatase does not seem to act on the nuclear nucleotides (Table IV).

The two experiments that have just been described show that phosphorylation of nucleotides occurs within the nucleus. The next experiments were arranged to enable us to compare nuclear phosphorylation with mitochondrial oxidative phosphorylation, which has been extensively investigated. An important characteristic of phosphorylation in mitochondria is that considerable quantities of nucleotides can be added to a mitochondrial preparation and that the nucleotides so added become phosphorylated. A distinctive feature of nuclear phosphorylation is that only intranuclear monophosphates seem to be phosphorylated, for added AMP is not acted upon. In an experiment to show whether nuclei act upon added nucleotides, thymus sucrose nuclei were isolated rapidly so that most nucleotides were still in the form of mononucleotides. At this point, to the concentrated nuclear suspension, about twice as much AMP as total intranuclear nucleotides was added with and also without the appropriate amount of inorganic phosphate. The suspension was then stirred for an additional 120 minutes in the cold. At the end of the experiment, exactly the same amount of AMP that had been added

TABLE III

Phosphorylation in Isolated Calf Thymus Sucrose Nuclei

Material analyzed	Total E_{260} in 1 gm. dry matter	Per cent of total E_{260} present as			
		AMP	ADP	ATP	Total adenine nucleotides
Sucrose nuclei after rapid isolation (25 min. at 2°C.)	308	19.0	10.5	7.6	37.1
Above nuclei stirred at 2°C. for 50 min. after isolation	308	3.5	5.7	24.5	33.7
Sucrose nuclei after rapid isolation (28° min. at 2°C.)	295	14.2	8.5	7.6	30.3
Above nuclei stirred gently at 2°C. for 60 min. after isolation	300	4.6	5.6	20.4	30.4
Calf thymus tissue	—	18.8	5.8	3.7	28.3
Sucrose nuclei prepared rapidly from above tissue (28 min. at 2°C.)	240	16.0	7.9	6.7	30.6
Above nuclei stirred gently at 2°C. for 60 min. after isolation	233	6.3	6.4	16.6	29.3

to the suspension was recovered as AMP, whereas the amount of ADP, and the amount of synthesized ATP were just the same in the material to which AMP had been added and in the control to which no AMP had been added. It should be noted that the phosphate required for phosphorylation is avail-

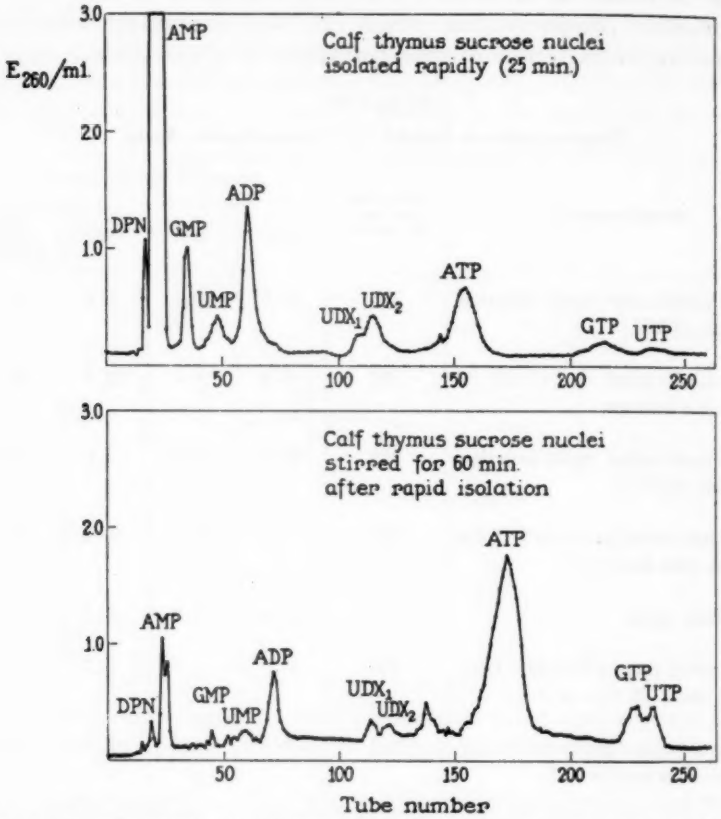

FIG. 4. Phosphorylation in isolated calf thymus sucrose nuclei. Acid-soluble nucleotides of sucrose nuclei isolated rapidly (in 25 minutes) at 2°C. (above), and of the same nuclei stirred gently in the cold for an additional 50 minutes (below). Note the increase in amount of triphosphates after 50 minutes.

able inside the nucleus; in no experiments on nuclear phosphorylation has the addition of phosphate been required. We have made some preliminary observations in this connection; sucrose nuclei when isolated rapidly contain less inorganic phosphate than is required for the phosphorylation of nuclear mononucleotides, and during the course of phosphorylation inorganic phosphate actually increases in amount. It is therefore probable that the phosphate available for the nuclear phosphorylation is derived from some organic phosphates in the nucleus.

When the effects of metabolic inhibitors are tested, one can see certain resemblances as well as differences between nuclear and mitochondrial phos-

TABLE IV

Synthesis of ATP in the Sucrose Homogenates of Calf Thymus Tissue with Separation into Nuclear and Cytoplasmic Fractions

Material analyzed	Per cent of total E_{260} present as					
	Unadsorbed to dowex 1	AMP	ADP	ATP	Total adenine nucleotides	Unadsorbed + total adenine nucleotides
Homogenate	18.0	14.5	3.1	1.5	19.1	37.1
Cytoplasm	28.0	7.7	2.3	1.0	11.0	39.0
Nuclei	18.0	2.5	3.2	14.4	20.1	38.1

TABLE V

Effects of Metabolic Inhibitors on Phosphorylation of Nucleotides in Isolated Calf Thymus Sucrose Nuclei

Inhibitor	Concentration	Change in phosphorylation due to added inhibitor	Inhibitory effect on		References for mitochondrial data
			Nuclear phosphorylation	Mitochondrial phosphorylation	
		per cent			
Sodium cyanide	1×10^{-3} M	−100	+	+	—
Carbon monoxide	100 per cent	−100	+	+	—
2,4-Dinitrophenol	2×10^{-4} M	−100	+	+	Loomis and Lipmann (1948)
Sodium azide	1×10^{-3} M	−100	+	+	Loomis and Lipmann (1949)
Antimycin A	1 μg./ml.	−61	+	+	Potter and Reif (1952)
Dicumarol	1×10^{-4} M	0	−	+	Martius and Nitz-Litzow (1953)
Janus green B	2×10^{-5} M	+6	−	+	Dianzani and Scuro (1956)
Methylene blue	2×10^{-5} M	+30	−	+	Lehninger (1949)
	2.5×10^{-4} M	+21			
Calcium ions	2×10^{-3} M	0	−	+	Lehninger (1949)
	3×10^{-3} M	0			
	4×10^{-3} M	0			

phorylation (Table V). Nuclear, like mitochondrial phosphorylation, is completely inhibited by some common inhibitors such as sodium cyanide, sodium azide (11), and dinitrophenol (12). Considering the mode of action of these inhibitors it is probable that the phosphorylation concerned is oxidative, and involves electron transport by means of cytochrome whereby energy-rich phos-

phate bonds are synthesized. Antimycin A (13) blocked this process by 60 per cent. Calcium ions are necessary in our system in order to maintain the nuclear structure. Varying the amount of calcium concentrations, from 2×10^{-3} M to $4 \times 10_0^{-3}$ M, did not influence the rate of phosphorylation. That no phosphorylation takes place in the cytoplasmic fraction under our experimental conditions has been mentioned already. Phosphorylation in the cytoplasmic fraction is probably prevented by the presence of calcium ions, a known potent inhibitor of mitochondrial oxidative phosphorylation (14). Certain reagents which block phosphorylation in mitochondria, namely dicumarol (15), Janus green B (16, 17), and methylene blue (14, 15), have no effect (if any, slightly accelerative) on nuclear phosphorylation. These observations may be taken as additional evidence against the participation of mitochondria in the phosphorylation occurring in preparations of isolated nuclei. In all inhibitor experiments, the adenine, uridine, and guanine nucleotides behaved in a parallel fashion.

Nuclei do not seem to contain the equipment for ordinary terminal oxidation as found in mitochondria, such as cytochrome *c* oxidase, cytochrome *c* and the flavoproteins including cytochrome *c* reductases (18, 19). In the case of calf thymus it has been reported that cytochrome *c* oxidase is sharply localized in the mitochondrial fraction; no detectable amount of this enzyme was found in the nuclei (19). The evidence that cytochrome *c* oxidase is absent from the nuclei is, however, perhaps not as decisive as would appear. The fact that the cytochrome *c* added to nuclei is not oxidized should be considered in relation to the fact that added AMP is also not acted upon. And yet in the latter case we know that nuclei actually phosphorylate the AMP already within them. It is, accordingly, worth considering that nuclei may contain cytochrome *c*, possibly in low concentration, which they are able to oxidize. Experiments on DPN cytochrome *c* reductase are unfortunately not decisive (19). If sucrose nuclei do not in fact contain any cytochrome *c* oxidase, it may be supposed that nuclear phosphorylation proceeds through a respiratory chain which differs from that present in mitochondria. One such difference might be the occurrence of different cytochromes, and in this connection it has already been shown that the cytochromes of microsomes differ from those of mitochondria (20, 21). Although inhibitor experiments indicate a difference between nuclear and mitochondrial phosphorylation, they do not yet throw any light on the details of the nuclear system.

Considering the fact that cyanide, azide, and dinitrophenol block nuclear phosphorylation the involvement of glycolysis in this process is unlikely.

Although most inhibitors which block nuclear phosphorylation also stop protein synthesis in the nucleus, at least one reagent, namely dicumarol, which does not affect phosphorylation does inhibit protein synthesis (1).

This result might indicate that dicumarol blocks the energy transfer from the nucleotide system to the synthesis of protein.

Retention of Nucleotides in the Nucleus.—The ability of sucrose nuclei to retain nucleotides is really quite surprising. It has been mentioned already that the bulk of the nucleotides is retained in the sucrose nuclei after their isolation. The nucleotides of isolated nuclei are well retained for at least 24 hours in the cold, or after repeated washings of nuclei with cold sucrose–$CaCl_2$ solution. Treatment of sucrose nuclei with 0.5 M sucrose, or with 0.25 M sucrose–0.003 M $CaCl_2$ containing 0.14 M NaCl has almost no effect on nucleotide retention.

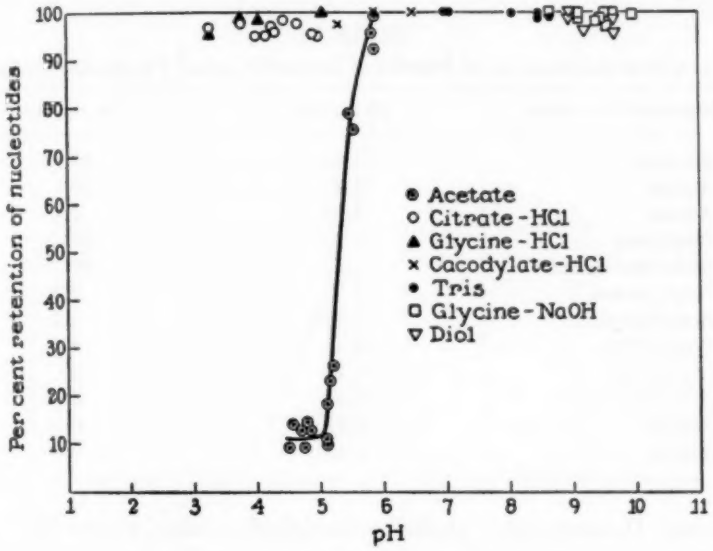

FIG. 5.

Nucleotide retention in the nuclei is, however, dependent on the presence of sucrose, for as soon as sucrose is replaced by a buffer solution in water at any pH, all nucleotides are completely lost from the nuclei. Citric acid nuclei, sucrose nuclei treated with 0.01 M citric acid without sucrose, or sucrose nuclei treated with saline in the absence of sucrose do not contain the nucleotides. One of the interesting features about nucleotide retention may be its irreversibility. Once nuclei lose their nucleotides by any means we have tried, nucleotides added to the medium cannot be taken up.

As long as there is sucrose in the medium, nuclei can be subjected to dilute buffer solutions over a wide pH range without loss of nucleotides (Fig. 5). In such experiments, to 1 volume of nuclear suspension, 9 volumes of 0.022 M buffer in 0.25 M sucrose–0.003 M $CaCl_2$ were added and kept in the cold for

30 minutes. After centrifuging, the pH and extinction at 260 mμ of the supernatant were measured. At least 95 per cent of nucleotides were retained over the whole pH range tested (pH 3.25 to 10.0). Buffers used included glycine-HCl, citrate-HCl, cacodylic acid–NaOH, "tris", "diol," and glycine-NaOH.

When sucrose nuclei were treated with a series of acetate buffers in sucrose solution, a surprising observation was made. Dilute acetate buffer (0.02 M) removed 85 to 90 per cent of the nucleotides from the sucrose nuclei below pH 5.1. In order to learn how specific acetate is in releasing nucleotides from nuclei in a sucrose medium, some related substances were tested. The results are included in Table VI. It is clear that certain simple fatty acids such as formic, propionic, and monochloroacetic have a similar ability to release

TABLE VI

Effect of Some Substances on the Retention of Nucleotides in Calf Thymus Sucrose Nuclei

Substances 0.02 M in sucrose	pH of media	Per cent loss
Formate	4.65	98
Acetate	4.45	90
Acetate	5.90	1.9
Propionate	4.32	95
Monochloroacetate	4.35	99
Ethyl alcohol	4.25	2.8
Acetaldehyde	4.40	4.1
Citrate-HCl	4.45	2.5
Glycine-HCl	4.10	0
Succinate	4.35	0
Acetone	4.35	0
Acetone	7.45	0

nucleotides. However, ethyl alcohol, acetaldehyde, citrate, glycine-HCl, succinate, and acetone have no effect on the removal of nucleotides from the sucrose nuclei.

As to the effect of acetate, it is obvious from Fig. 5 that there is a sharp boundary of effective pH of acetate between pH 5.1 and pH 5.9. In this pH range, the quantity of nucleotides released by acetate gradually increases as the pH drops. At pH 5.9 there is no loss of nucleotides by acetate; at pH 5.1 the maximum loss occurs.

A time course study on the effect of dilute acetate (0.005 M) at pH 4.5 (see Fig. 4) revealed that 90 per cent of the nucleotides were released within less than 2 minutes without further increase over 30 minutes; at pH 5.9 there is no sign of nucleotide release by acetate even after 30 minutes. These facts clearly indicate that release of nucleotides from the nuclei by acetate is pH-dependent. Release of nucleotides by propionic acid, formic acid, or monochloroacetic acid shows the same pH dependence as acetate (Fig. 7). Since

the dissociation constants of formic, acetic, propionic, and monochloroacetic acids differ considerably from each other, the effect of pH on release of nucleotides seems to be due not to the effect of pH on the reagents added but rather to the effect of pH on a component of the nuclei with which acetate and the other substances react.

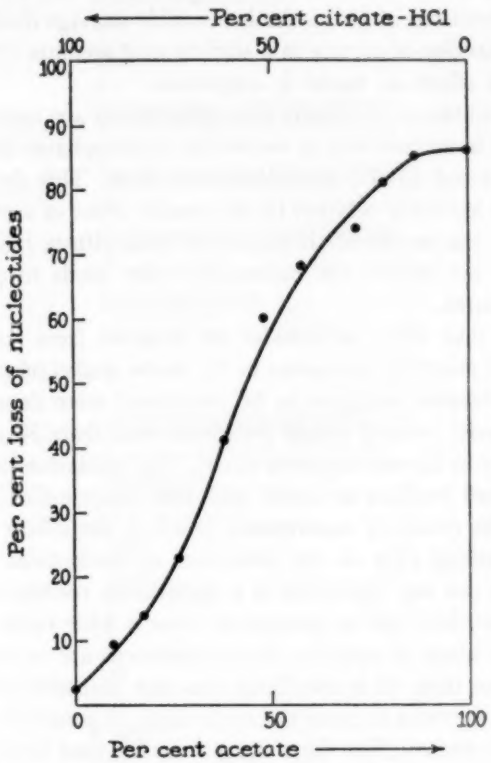

FIG. 6. Effect of varying acetate: citrate ratio on nucleotide retention in calf thymus sucrose nuclei.

To study the quantitative relationship between acetate and the removal of nucleotides from the nuclei, buffers were prepared containing a mixture of acetate and citrate at pH 4.5 in which the total molar concentration was kept constant but in which the acetate concentration was varied. It is evident from Fig. 6 that increasing concentration of acetate results in a corresponding increase in amount of nucleotides removed from the nuclei. In other experiments the amount and concentration of acetate were held constant but the amount of nuclear material was varied over a threefold range. It was found that the quantity of nucleotides released was proportional to the amount of

nuclear material present. Under these conditions, therefore, the concentration of acetate is the determining factor in releasing the nucleotides at the effective pH even when relative amounts of acetate and nuclei are varied. In further experiments on the acetate effect, we are planning to use C^{14}-labelled acetate to study the combination of acetate with nuclear constituents.

Our colleague, Dr. R. Lorente de Nó, has called our attention to some remarkable observations of his on the irreversible damage done to nerve fibers by a low concentration of acetate in a slightly acid medium (22). The analogy with the acetate effect on nuclei is suggestive.

It should be mentioned in passing that when nuclei are treated with acetate buffer at pH 4.5 in sucrose–$CaCl_2$, nucleotide triphosphates and diphosphates are largely converted to the monophosphate form. This dephosphorylation does not seem to have any relation to the specific effect of acetate in releasing nucleotides from the nuclei, for it was found that citrate buffer at the same pH (which does not release the nucleotides) also tends to dephosphorylate tri- and diphosphates.

It was found that when nucleotides are released from sucrose nuclei by acetate and other reagents, potassium in the nuclei is also released at the same time. All the potassium analyses to be mentioned were done by Dr. Shinji Itoh. Sucrose nuclei usually retain somewhat less than 50 per cent of the potassium present in the non-aqueous nuclei. This potassium is well preserved even after repeated washing of nuclei with cold sucrose–$CaCl_2$ solution (23). Fig. 7 includes the results of experiments in which the effects of a number of substances at various pHs on the retention of nucleotides and potassium were tested. One can see that there is a remarkable correlation between the retention of nucleotides and of potassium; over a wide range of pH as well as in the various kinds of reagents, both substances are in each instance released at the same time. It is not likely that the potassium released is combined with the nucleotides because the molar ratio of potassium to nucleotides removed is about twenty. The facts suggest rather that both substances are part of a common complex in the cell nuclei; acetate and other effective reagents disturb this complex in some way so that nucleotides and potassium are released.

We now have reasons to suppose that the nucleotides of the nucleus are held in some complex structure and that only such nucleotides are involved in the metabolism of the cell nucleus. The evidence for this is: (1) nucleotides are firmly held in the nucleus under a variety of conditions, and some special reagents such as acetate can release them together with potassium from the sucrose nuclei; (2) once nuclei lose their nucleotides, added nucleotides are not taken up; (3) only intranuclear nucleotides are phosphorylated—added nucleotides cannot be acted upon; (4) added AMP, ADP, or ATP have no effect on the synthesis of protein by isolated sucrose nuclei, whereas upon

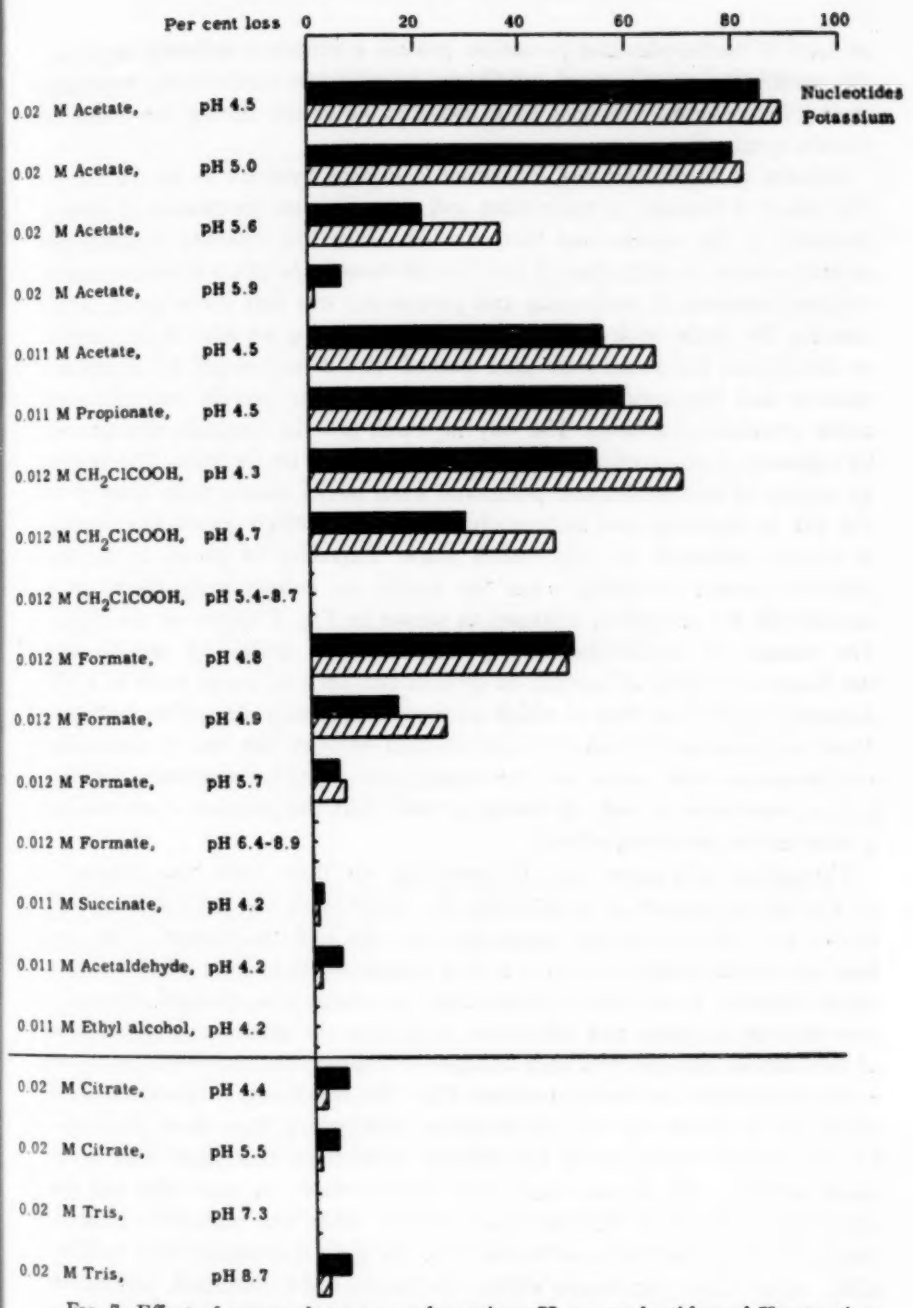

FIG. 7. Effect of some substances and varying pHs on nucleotide and K retention in calf thymus sucrose nuclei.

removal of nucleotides and potassium protein synthesis is seriously impaired; (5) under our experimental conditions intranuclear nucleotides, especially their triphosphates, are held to a considerable extent during the course of protein synthesis.

Relation of Nucleotides and Potassium to Protein Synthesis in the Nucleus.— The effect of removal of nucleotides and potassium on the process of protein synthesis in the nucleus has been studied. Since this involves exposing the nuclei to media in which the pH has been decreased, the effect of such exposure (without removal of nucleotides and potassium) was first investigated. After treating the nuclei with a sucrose solution containing an acid buffer (citrate or cacodylate) the nuclei were then washed with a sucrose–pH 6.7 phosphate mixture and the uptake of C^{14}-alanine into nuclear protein was measured under standard conditions. The way in which protein synthesis was reduced by exposure to acid media is shown Fig. 8 (the curve on the left). The release by acetate of nucleotides and potassium from nuclei occurs quite sharply as the pH is decreased and fortunately the point at which severe inactivation of protein synthesis by acid takes place. Exposure of nuclei to acetate prevents protein synthesis, when the nuclei are subsequently placed in a sucrose–pH 6.7 phosphate mixture, as shown in Fig. 8 (curve on the right). The release of nucleotides and potassium from nuclei by acetate and the destructive effect of acetate on protein synthesis in nuclei occur at a pH distinctly higher than that at which acidity *per se* greatly diminishes synthesis. These experiments demonstrate a correlation between the loss of nucleotides and potassium from nuclei and the impairment of protein synthesis in nuclei. Such a correlation is not, of course, a proof that the presence of nucleotides is required for protein synthesis.

Throughout this paper and the preceding one there have been comments on the role of sucrose in maintaining the integrity of the nucleus. It is well known that the microscopic appearance (in the light microscope) of an isolated nucleus is different when it is in a sucrose medium from what it is in a saline medium. If in a given preparation the medium is changed repeatedly from sucrose to saline and then back to sucrose the microscopic appearance of the nucleus changes with each change of medium; the alterations in microscopic appearance are readily reversed (24). The conditions within the nucleus which are preserved by sucrose solutions, and which have been significant for the present investigation, are different from those that have been recognized hitherto. The intranuclear conditions to which we now refer and the maintenance of which depends upon sucrose, when the nucleus is isolated, cannot be recognized by examination with the light microscope; and, furthermore, when these conditions within the nucleus are disturbed, restoration has not been possible. We are here dealing with what we must suppose are changes in the fine structure of the nucleus, changes which affect the metabolic activity of the nucleus.

That intranuclear conditions depend upon keeping isolated nuclei in a sucrose medium is shown by the following facts: (1) synthesis of protein stops if sucrose is removed and is not restored if the nuclei are again placed in a

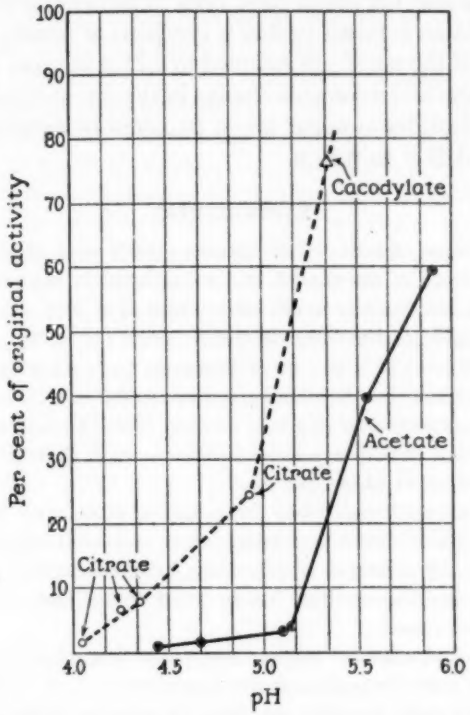

FIG. 8. Effect of the exposure of calf thymus sucrose nuclei to acidic buffer solutions on the subsequent synthesis of protein. Sucrose nuclei were exposed to the buffers in sucrose–$CaCl_2$ indicated in the figure. After 30 minutes, the nuclei were washed with 0.02 M sodium phosphate buffer in sucrose–$CaCl_2$ at pH 6.7, and tested for the incorporation of C^{14}-alanine into the nuclear proteins. Loss of nucleotides by the acidic buffer treatment from the nuclei was: in the citrate and cacodylate series, reading points on the curve from left to right, 6.1, 6.1, 5.1, 6.1, and 2.3 per cent of the control; in the acetate series, reading points on the curve from left to right, 90.5, 90.5, 84.0, 77.5, 26.5, and 9.3 per cent of the control. Abscissa represents the pH of the buffer solution measured after the treatment of nuclei.

sucrose medium; (2) mononucleotides are released when isolated nuclei are placed in a medium not containing sucrose and nucleotides are not again taken up by the nuclei when they are returned to a sucrose medium; (3) when sucrose nuclei are treated with DNAase they lose as much as 80 to 90 per cent of their DNA and yet only 15 per cent of their histone is released, although histone, a water-soluble protein, is attached to DNA in the nucleus; on the

other hand, when nuclei are in a saline medium (containing no sucrose) at the same time that DNA is removed by the action of DNAase about 50 per cent of the histone is released from the nuclei.

In our preparations the nuclei have been in isotonic sucrose, 0.25 M. If the sucrose concentration is raised to 0.50 M synthesis of protein no longer occurs, nor does it occur if the nuclei are returned to 0.25 M sucrose; apparently hypertonic sucrose causes an irreversible change in the fine structure of the nucleus. Retention of nucleotides in nuclei is not impaired by raising the sucrose concentration from 0.25 M to 0.50 M.

EXPERIMENTAL

Calf Thymus Sucrose Nuclei.—Calf thymus glands were obtained as quickly as possible after the death of the animal, and transported to the laboratory in ice cold 0.25 M sucrose solution. Sucrose nuclei were prepared in 0.25 M sucrose containing a small amount of $CaCl_2$ as has been described before (1, 2). In earlier experiments, 0.25 M sucrose–0.002 M $CaCl_2$ was used. However, to prevent formation of nuclear gels, Ca^{++} concentration has been increased up to 0.003 M in more recent experiments. All operations were done in a cold room at 2°C. The final volume of a nuclear preparation suspended in 0.25 M sucrose–$CaCl_2$ was such that the dry weight of the suspension was from 35 to 50 mg. per ml.

Non-Aqueous Nuclei.—Preparations from calf thymus, calf liver, and chicken erythrocytes as well as their corresponding whole tissues or cells used in this study were those described by Allfrey *et al.* (3), which had been kept at −20°C. until used.

Trout Sperm Cells.—Frozen dried brown trout sperm and rainbow trout sperm stored at −20°C. were used.

Dry Weight.—2 ml. of a sucrose nuclear suspension was centrifuged at 4,000 R.P.M. for 5 minutes in the cold. The sediment was suspended in 7 ml. of 88 per cent alcohol, and kept in a water bath at 60°C. for 5 to 10 minutes. After centrifugation, the sediment was incubated in the same way as before with 88 per cent alcohol twice, and 95 per cent alcohol twice. It was then washed with 7 ml. of ether twice, and dried at 110°C. All centrifugations were done at room temperature. It is necessary to wash the sucrose nuclei with hot 88 per cent alcohol for the complete removal of the sucrose present. Weighed samples of fresh tissue and non-aqueous preparations were treated with 95 per cent alcohol three times, and with ether twice. They were then dried at 110°C. and weighed again.

Preparation of an Acid-Soluble Extract for the Chromatographic Fractionation of Nucleotides.—All operations were carried out in a cold room at 2°C. To 8 volumes of nuclear or tissue suspension, 2 volumes of 10 per cent perchloric acid were added, and stirred for about 10 minutes. The precipitated protein was sedimented at 5,000 R.P.M. for 5 minutes, and extracted again with 5 volumes of 2 per cent perchloric acid. The combined supernatant was brought to pH 6 to 7 by careful dropwise addition of 6 N KOH. The precipitate of $KClO_4$ was removed by centrifugation. Part of the supernatant was used for an extinction measurement at 260 mμ. A large aliquot was used for the chromatographic analysis of nucleotides.

Chromatographic Fractionation of Nucleotides.—The "formic acid system" of

dowex 1–formate of Hurlbert *et al.* (4) was used throughout this investigation. The elution system was adapted for the fractionation of relatively small amounts of samples by using a 100 ml. reservoir, a 50 ml. mixing flask, and a 0.6 × 20 cm. dowex 1–formate resin column. Adsorbed nucleotides were eluted with a continuously increasing concentration of eluant, that is, first with 100 ml. of 4 N formic acid, followed by successive 100 ml. volumes of 0.2 M ammonium formate in 4 N formic acid, 0.4 M ammonium formate in 4 N formic acid, and 1 M ammonium formate in 4 N formic acid. Samples of effluent containing 1.3 ml. for a 15 minute flow were collected automatically. The extinction at 260 mμ was measured for each tube to determine the quantity of nucleotide present. The extinction at 275 mμ was also measured for an approximate characterization of each peak. Each nucleotide peak was identified by its location on the dowex 1–formate chromatograms, and also after lyophilization the material in each peak was identified by its ultraviolet spectrum, orcinol reaction, diphenylamine reaction, etc. (*cf.* Hurlbert *et al.* (4)).

Postmortem Changes of Nucleotides in Rat Thymus Tissue.—The rats were kindly given to us by Dr. John B. Nelson of The Rockefeller Institute. Twelve individuals, age 8 to 9 weeks, were killed by decapitation, and their thymus glands were removed. Some of them were immersed in liquid nitrogen immediately upon removal, and lyophilized. Dried thymus corresponding to 1 gm. of wet weight was ground in a mortar with 10 ml. of cold 2 per cent perchloric acid. The acid-soluble extract was prepared as described before. 1 gm. samples of freshly excised thymus tissue were incubated in glass tubes in a water bath at 30°C. for 5, 10, and 20 minutes. After incubation, 10 ml. of cold 2 per cent perchloric acid was added to each sample and an acid-soluble extract was prepared. All samples were chromatographed on a dowex 1–formate column. The average weight of the thymus from rats of the ages used was 0.33 gm.

Phosphorylation in Isolated Nuclei.—A sucrose nuclear preparation was made quickly (in 26 minutes) from 50 gm. of calf thymus, and finally suspended in 35 ml. sucrose–$CaCl_2$. To a 15 ml. aliquot of the nuclear suspension 4 ml. of 10 per cent perchloric acid was immediately added and an acid-soluble extract was prepared. Another 15 ml. sample was stirred gently with a mechanical stirrer for 50 minutes at 2°C. At the end of this time, another acid-soluble extract was made. Both samples were chromatographed.

Phosphorylation in a Calf Thymus Homogenate.—140 gm. of calf thymus was minced and homogenized with 630 ml. of 0.25 M sucrose–0.003 M $CaCl_2$ in a low speed blendor (1,300 R.P.M.) for 4 minutes. The homogenate was strained through gauze and then through flannelette. The volume of the strained fluid was 580 ml. A perchloric acid extract was immediately prepared by adding 60 ml. of 10 per cent perchloric acid to 250 ml. of this homogenate. Another 250 ml. sample was allowed to stand for 95 minutes at 4°C. with gentle mechanical stirring, and was then centrifuged for 7 minutes at 3,000 R.P.M., the supernatant being the cytoplasmic fraction. This was centrifuged again to remove the nuclei still present. The nuclear sediment from the first centrifuging was washed once with cold sucrose–$CaCl_2$. The supernatant was combined with the cytoplasmic fraction. Acid-soluble extracts were prepared from both nuclear and cytoplasmic fractions. All acid-soluble extracts were analyzed chromatographically for nucleotides.

Phosphorylation in Presence of Added AMP.—Calf thymus sucrose nuclei were prepared quickly and suspended in 35 ml. of sucrose–$CaCl_2$ solution. To 10 ml. aliquots were added: (1) 1.63 ml. 0.25 M sucrose containing 5 mg. AMP (pH 6.8); (2) 1.84 ml. 0.025 M sodium phosphate buffer at pH 6.8 in 0.25 M sucrose containing 5 mg. AMP; (3) 2.0 ml. 0.25 M sucrose. They were then stirred gently for 120 minutes at 20°C. The acid-soluble extract of each sample was analyzed for nucleotides on a dowex 1–formate column.

Inorganic Phosphate.—Analyses of inorganic phosphate in the sucrose nuclei present a difficulty because of the presence in the 2 per cent perchloric acid extract of lysine-rich histones which form a heavy precipitate with ammonium molybdate. In this study both of the following ways were adopted.

(*a*) 10 ml. of a cold 2 per cent perchloric acid extract of sucrose nuclei was carefully neutralized with 6 N KOH, and insoluble $KClO_4$ was removed by centrifugation. The clear supernatant was then dialyzed in a cellophane bag against distilled water overnight in a cold room. The volume of outside plus inside fluid was 20 ml. Inorganic phosphate was determined on an aliquot of outside fluid.

(*b*) 20 ml. of cold 2 per cent perchloric acid extract of sucrose nuclei was neutralized with 6 N KOH, followed by the centrifugation of the $KClO_4$ precipitate in the cold. The supernatant was then chromatographed on a dowex 1–formate column. A peak of inorganic phosphate was lyophilized. The dry material was dissolved in 10 ml. of water. Inorganic phosphate was analyzed on an aliquot of this solution.

Effect of Inhibitors on Nuclear Phosphorylation.— Effect of NaCN, 2,4-dinitrophenol, sodium azide, dicumarol, Janus green B (National Aniline Division, CI No. 133), methylene blue (Grübler, Leipzig), antimycin A.—About 150 gm. of calf thymus tissue was grossly minced. 50 gm. of this was further finely minced in a sucrose-$CaCl_2$ solution containing an appropriate amount of one of the above mentioned inhibitors. Nuclei were kept in the presence of inhibitor throughout their isolation. The isolated nuclei were suspended in 15 to 20 ml. of sucrose–$CaCl_2$ containing an inhibitor, and allowed to stand for 60 minutes. The concentration of each inhibitor was kept constant throughout the experiment. Control nuclei were prepared in parallel from 50 gm. of minced tissue and treated in the same way in the absence of inhibitor. Acid-soluble extracts were chromatographically analyzed for nucleotides. The extent of inhibition of phosphorylation of nuclear AMP was calculated from the per cent of the total extinction at 260 mμ of ADP and ATP (ADP as 1, and ATP as 2) found in the experimental and control series. The per cent of the total extinction at 260 mμ of ADP and ATP at the beginning of the experiment was estimated on the whole tissue, because it has been shown by the analyses of non-aqueous preparations that the relative concentration of AMP, ADP, and ATP in whole tissues and in the nuclear fraction is the same.

Effect of Buffers at Different pHs, and of Some Reagents on the Retention of Nucleotides and Potassium in Sucrose Nuclei.—To 1 ml. of sucrose nuclei 9 ml. of 0.022 M buffer solutions in 0.25 M sucrose–0.003 M $CaCl_2$ was added, stirred, and allowed to stand for 30 minutes at 2°C. The suspension was centrifuged at 5,000 R.P.M. for 5 minutes. As control 9 ml. of sucrose–$CaCl_2$ without buffer was used. The pH of each supernatant while cold was determined with a glass electrode. 1 ml. of 10 per cent perchloric acid was added to 4 ml. of supernatant to precipitate protein if present.

The extinction at 260 mμ was read on the clear perchloric acid supernatant, and also on the 2 per cent perchloric acid supernatant obtained directly from 1 ml. of original nuclei. Acetaldehyde, alcohol, acetone, succinic acid, propionic acid, and monochloroacetic acid were used in a desired concentration by mixing with citrate buffer–sucrose–$CaCl_2$. The pH of each solution was adjusted with 1 N NaOH or HCl before use. The effects of these substances were tested under the conditions mentioned above.

Potassium analyses were kindly performed for us by means of a flame photometer by Dr. Shinji Itoh on the residue obtained when the sucrose suspensions of nuclei were centrifuged in the experiments described above.

Effect of the Removal of Nucleotides and Potassium by Acetate from Sucrose Nuclei on the Synthesis of Protein.—Each 10 ml. sample of calf thymus sucrose nuclear suspension was centrifuged down at 3,000 R.P.M. for 7 minutes at 2°C. The sediments were then taken up to 25 ml., each with one of the following solutions: *control series* (1) 0.25 M sucrose–0.003 M $CaCl_2$, (2) citrate buffer pH 4.0, (3) citrate buffer pH 4.2, (4) citrate buffer pH 4.5, (5) citrate buffer pH 5.0, (6) cacodylate buffer pH 5.4; *acetate series*- acetate buffer (1) pH 4.0, (2) pH 4.45, (3) pH 4.75, (4) pH 4.95, (5) pH 5.3, (6) pH 5.7. All buffers were in 0.25 M sucrose–0.003 M $CaCl_2$ at the concentration of 0.02 M. Each suspension was then allowed to stand for 30 minutes in the cold. After centrifugation at 3,000 R.P.M. for 7 minutes, the pH and the extinction at 260 mμ were determined on each supernatant (for procedure see preceding section). The residue was washed with 25 ml. of 0.02 M sodium phosphate buffer at pH 6.7 in sucrose–$CaCl_2$. 1 ml. of each nuclear suspension was used for the C^{14}-alanine incorporation experiments as described in the previous paper (1). The extent of C^{14}-alanine incorporation by each nuclear preparation was expressed as "per cent of original activity," taking the preparation (1) in the control series as 100.

SUMMARY

1. It has been demonstrated by ion exchange chromatography that the cell nucleus contains mononucleotides of adenine, guanine, cytosine, uracil, together with diphosphopyridine nucleotide, and several uridine diphosphate derivatives; the adenine nucleotides predominating in amount. Nucleotide components in the cell nucleus are in close agreement both quantitatively and qualitatively with those found in the cytoplasm.

2. In calf thymus sucrose nuclei, nucleotide monophosphates can be phosphorylated to the energy-rich triphosphate form *without* participation of cytoplasmic components. As to the nature of the phosphorylation, it has been shown that there exist certain differences as well as resemblances between nuclei and mitochondria. A distinctive feature of nuclear phosphorylation is that only intranuclear monophosphates seem to be phosphorylated. The process is completely inhibited by cyanide, azide, and dinitrophenol. However, certain reagents which block oxidative phosphorylation of mitochondria, namely dicumarol, Janus green B, methylene blue, and calcium ions, have no effect on phosphorylation within the nucleus.

3. The bulk of mononucleotides is preserved within thymus nuclei after their isolation in sucrose. Nucleotides are surprisingly well retained by nuclei in a sucrose medium whether or not electrolytes are present and in buffers ranging from pH 3 to 10; under all conditions sucrose is required for retention.

4. Dilute acetate in sucrose releases nucleotides from the nucleus below pH 5.1. As to the effective pH of acetate, there is a sharp boundary between pH 5.1 and pH 5.9. At pH 5.9, and above, acetate does not remove nucleotides from the nucleus. The effects of propionate, formate, and monochloroacetate on the nuclei are the same as that of acetate.

5. When nuclei are exposed to a wide variety of conditions a close correlation is found between the retention in the nucleus of nucleotides and of potassium. This suggests that both substances are part of a common complex in the cell nucleus.

6. It has been shown that upon removal of nucleotides and potassium from calf thymus sucrose nuclei by acetate, the ability to incorporate C^{14}-alanine into nuclear protein is greatly impaired.

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NARCOSIS AND EMULSION REVERSAL BY INERT GASES*

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(Received for publication, October 30, 1956)

INTRODUCTION

This work represents a continuation of a series of studies from this laboratory in which it was shown that nitrogen and other inert gases at sufficient pressures are able to exert a true narcotic effect (1–4). Although this was first demonstrated only in reactions involving a synapse it also occurs with higher pressures in peripheral nerve. The present experiments were initiated in an effort to elucidate further the mechanism of this narcotic effect exerted by a chemically inert molecule. Clowes (5) showed that Ca, alcohols, and certain other narcotics are able to reverse emulsions stabilized with sodium soaps from the oil in water to the water in oil state and Hirschfelder and Serles (6) have used this model to explain the antagonistic action of Ca and Mg. We proposed to determine whether reversal could also be observed with the inert gases.

Experimental Procedure

As suggested by Clayton (7) resistance to current flow was used to determine the state of the emulsion. When water is the continuous phase, the emulsion will conduct a current provided a sufficient concentration of an electrolyte is present, whereas if the oil phase is continuous, little or no current will flow.

The emulsion was prepared in a glass-lined[1] steel chamber which had a volume of 30 ml. Through the wall of the chamber were inserted two platinum electrodes through which the resistance was determined with a Wheatstone bridge and direct current while the emulsion was being shaken. In later experiments we used alternating current and found no essential difference in the values obtained.

The emulsions formed consisted of 5 ml. of olive oil and 5 ml. of 4 mM NaOH solution. The exact volume ratio did not seem to be critical in determining the state of the emulsion. The free fatty acid present in the oil reacted with the NaOH to form soap which stabilized the oil in water emulsion. The shaking of the emulsion was standard-

* These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and The University of Rochester, NR 110-281.

[1] We are indebted to the Pfaudler Company of Rochester, New York, for their courtesy in applying the glass lining to the chamber.

ized as to time and amplitude with a Burrell wrist action shaker. Before use the various gases were transferred to smaller tanks containing 50 ml. of 1 M NaOH to absorb any traces of CO_2 present.

Controls were alternated with the experimental emulsions. They consisted of emulsions formed in the same way and subjected to exactly the same conditions except for the pressure of gas.

We standardized the procedure as follows: shake at maximum amplitude for 5 minutes; add the gas; shake for 10 minutes; allow to stand for 10 minutes; then shake for 5 minutes. The resistance was measured at definite times during the procedure, and any pressure of gas was considered below the threshold if it did not reverse the emulsion by the end of the last period of shaking. All experiments were run at room temperature.

RESULTS

Prior to any experiments with gases we determined the amounts of $CaCl_2$ required to reverse oil in water emulsions formed with varying amounts of NaOH. The purpose was to characterize an emulsion which was definitely oil in water, but which required a minimum of $CaCl_2$ to effect reversal. The data shown on Fig. 1 were obtained from emulsions of two different volumes (4 ml. and 20 ml.) consisting of equal parts of oil and water.

The dotted line shows the chemical equivalence of calcium and sodium. The amount of calcium required to reverse fell somewhat below this line. Since pure olive oil and water when shaken vigorously together give a water in oil emulsion, we felt that a minimum amount of Na was necessary to stabilize an oil in water emulsion and that the reduction of the Na concentration by Ca to this value would cause reversal before all the Na was replaced. Calcium apparently replaces sodium in combination with free fatty acids and when all the soap has thus reacted further addition of sodium is without effect. This explains then the plateau reached in Fig. 1. The fact that the two graphs at two different volumes did not reach a plateau at the same concentration of Ca may possibly be attributed to differences in the intensity of shaking and in the oils used.

As a result of these preliminary experiments we decided to use emulsions stabilized with 0.004 M NaOH, since this was sufficient to prevent autoreversal without making the emulsion insensitive to reversing agents.

Table I summarizes the effects of the various gases on the state of such emulsions. No reversals were obtained with helium at the highest pressures available. Sixteen experiments with N_2O at pressures up to 53 atmospheres did not cause reversal. Higher pressures were not available since the gas liquefies at 71.8 atmospheres at 21°C. Nitrogen required pressures above 100 atmospheres while argon seemed to be effective at pressures above 60 atmospheres. While the greater effectiveness of argon compared to nitrogen seems well established by these data, the determination of the exact magnitude of the difference would require further experimentation.

Carbon dioxide was very effective in reversing the emulsions. To measure the amounts required we placed emulsions with 30 ml. of oil and 30 ml. of either 4, 8, or 16 mM/liter NaOH in a flask which was then evacuated to a

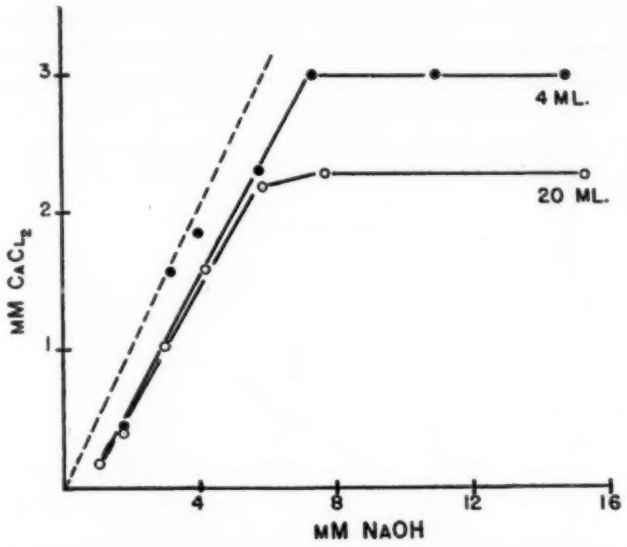

FIG. 1. Concentrations of $CaCl_2$ required to reverse oil-in-water emulsions at different concentrations of NaOH. Two experiments with 4 and 20 ml. of emulsion, respectively.

TABLE I

State of Emulsion at Different Partial Pressures of Inert Gases

Gas	Pressure	No. of cases	
		Water in oil	Oil in water
	atmospheres		
Helium	80–107	0	9
Nitrous oxide	7–53	0	16
Nitrogen	130	5	2
	100	5	3
	67–87	2	5
	40	0	3
Argon	60–80	11	0
	33–53	2	11

pressure of 30 to 50 mm. Hg at room temperature. When CO_2 was introduced into the flask in small amounts the pressure first increased promptly and then fell slowly toward its previous level as CO_2 was absorbed by the emulsion. By recording the initial and final pressures we were able to determine

the amounts of CO_2 absorbed and the final pressure of the gas. If this amount failed to reverse the emulsion more CO_2 was added and so on until it was visually apparent that the emulsion had reversed.

These data are shown in Fig. 2. The upper points (hollow circles) represent the average total amounts of CO_2 which went into the emulsion, the standard error is represented by the bars. The lower points (solid circles) are the

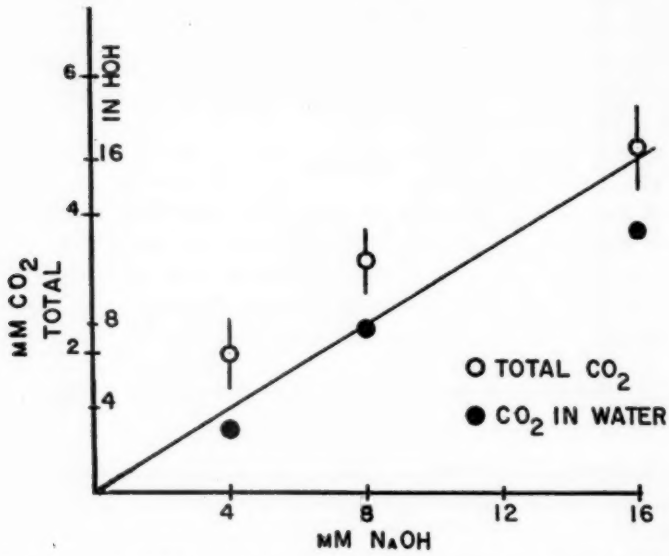

FIG. 2. Amounts of CO_2 required to reverse the emulsion at different concentrations of NaOH (millimols per liter) in the water phase. Ordinates at the left represent the total millimols of CO_2 absorbed by 600 cc. of the emulsion at the reversal point. Ordinates at right (solid dots) represent calculated concentrations of CO_2 in water phase in millimols per liter. The solid line represents equal concentrations of NaOH and CO_2 (as CO_2 or as HCO_3^-) in the water phase.

amounts calculated by difference to be in the water phase on the assumption that the oil phase is saturated at the partial pressure of CO_2 remaining after equilibration. The straight line is drawn through the points of chemical equivalence of HCO_3^- and Na^+. It is evident that reversal occurs approximately at the point where all the Na is in the form of $NaHCO_3$.

DATA

DISCUSSION

Bancroft's theory (8) of emulsions states that if two immiscible liquids are shaken together the one with the lower interfacial tension will surround the one with the higher. Or in other words, the phase with the higher tension

will round itself into a sphere—the position with the lowest surface energy—and the other phase will, as a result, surround it. This state can be changed if the interfacial tension of the dispersed phase is lowered below that of the continuous phase.

By occupying space in the surface of either the water or the oil phase, an inert particle could lower the interfacial tension and this would reverse the emulsion if the amount accumulated in the dispersed phase were sufficient to lower its interfacial tension below that of the continuous phase.

The accumulation of an inert gas molecule at the interface would be expected according to Roberts' postulation that the absorption of a substance

TABLE II

Gas	Oil/water solubility 37°C.	Oil solubility Bunsen coefficient 37°C.	Emulsion reversal pressure
			atmospheres
Argon*	5.3	0.14	60
Nitrogen*	5.2	0.067	100
Nitrous oxide	3.2‡	1.6§	53+
Helium*	1.7	0.015	107+
Carbon dioxide	1.6‖	0.876‖	0.01

Sources of data for table:

* Behnke, A. R., and Yarbrough, O. D., *Am. J. Physiol.*, 1939, **126**, 409.

‡ Adriani J., The Chemistry of Anaesthesia, Springfield, Illinois, Charles C Thomas, 1946, 62.

§ Carpenter, F., *Am. J. Physiol.*, 1954, **178**, 507.

‖ Cotton seed oil at 40°C.

at an interface will increase as the ratio of electrical charge/mass for that substance decreases (9).

According to these ideas any inert gas would be expected to reverse an emulsion if it possessed two properties: (1) a tendency to partition itself into the oil phase rather than the water phase, and (2) the ability to lower the interfacial tension of the oil below that of the surrounding water.

Comparison of the solubility characteristics of the gases used is given in Table II.

From column I we would expect the order of effectiveness of gases in reversing an emulsion to be Ar > N_2 > N_2O > He > CO_2. Argon actually did seem to have a lower threshold than N_2. Since the maximum pressure of N_2O available is 53 atmospheres it was not possible to determine whether its emulsion reversal threshold really exceeds those of argon and nitrogen but it was certainly closer to these than would be expected from its oil solubility. The ineffectiveness of N_2O at 53 atmospheres is explainable from the oil/water solubility ratio, but from oil solubility alone N_2O would be expected to be the most effective of the gases used.

Helium did not cause reversal at pressures where N_2 was effective. Since higher pressures of helium were not available it was not possible to determine whether helium at any pressure will reverse an emulsion, but certainly it is less effective than nitrogen or argon. So far as they go, these data therefore support the parallelism between effectiveness of emulsion reversal and oil/water solubility ratios for Ar, N_2, and He. The data for nitrous oxide are not in conflict with this theory but are definitely not in accord with the oil solubility order.

The great effectiveness of CO_2 we attributed to its chemical action since from the oil/water solubility ratio it should require greater pressures than helium before causing reversal, and from the oil solubility data it should be second to nitrous oxide in effectiveness.

These experiments suggest therefore that narcosis may be produced by accumulation of the inert gases in the lipide in excess of the amount in the watery components of the membrane and in amounts sufficient to render the lipide phases more continuous; thus the membrane becomes stabilized and a region such as the synapse does not conduct an impulse.

CONCLUSION

Investigations of the effect of high pressures of N_2 (100 to 130 atmospheres) and of Ar (60 to 80 atmospheres) showed that these gases are effective in reversing the phases of an oil in water emulsion. Nitrous oxide did not cause reversal at pressures as high as 53 atmospheres nor did helium as high as 107 atmospheres. We found CO_2 most effective in reversing the emulsions and attributed this to its chemical properties. It is suggested that these observations may help to explain the narcotic effects of inert gases.

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THE EFFECT OF ULTRAVIOLET LIGHT ON THE PRODUCTION OF BACTERIAL VIRUS PROTEIN*

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(Received for publication, September 4, 1956)

Hershey and Chase discovered in 1952 (1) that it is the deoxyribonucleic acid (DNA) and not the protein of bacteriophage T2 which is responsible for the initiation of phage reproduction within an infected bacterial cell. The phage DNA, therefore, appears to have at least two roles in the synthesis of the progeny bacteriophage particles: one is to cause its own, several hundredfold replication and the other is to induce the formation of phage-specific protein. The present work is an attempt to study the second of these roles by examining to what extent and how long after infection the integrity of the phage DNA is required for the successful synthesis of that phage-specific protein whose presence can be detected by virtue of its ability to react with antiphage serum (2, 3). For this purpose, the effect of ultraviolet light (UV) irradiation on the appearance of protein possessing phage antigenicity inside T2-infected bacterial cells has been studied at various stages of phage development.

Materials and Methods

Bacteriophage T2r$^+$ and its host *Escherichia coli*, strain B(H), were used in this study.

Tris-glucose medium (4) contains per liter 2 gm. glucose, 5.4 gm. NaCl, 3 gm. KCl, 1.1 gm. NH_4Cl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 M tris-HCl buffer (tris-hydroxymethyl amino methane) adjusted to pH 7.2 at 37°C., 20 mg. phosphorus (as KH_2PO_4), and 6 mg. sulfur (as Na_2SO_4). In this medium, the latent period of T2 is 22 minutes at 37°C.

The general methods of bacterial culture, preparation of phage stocks and antisera, and assay of infectivity used in this study were those described by Adams (5).

Determination of Phage Antigen.—T2-antigenic protein has been defined here as that protein which can be precipitated specifically with anti-T2 rabbit serum. The amount

* Supported by a grant from the National Cancer Institute of the National Institutes of Health, United States Public Health Service to Dr. W. M. Stanley and Dr. G. S. Stent.

‡ On leave from the Institute of Science and Technology, University of Tokyo (1953–1955).

of protein so precipitated was determined by measurement of the radioactivity in precipitates (3) formed by the addition of anti-T2 serum to S^{35}-labelled lysates, according to the following procedure: The crude radioactive lysate was first dialyzed in the cold against adsorption buffer (1) (containing 0.003 M KCN and 20 mg./ml. chloramphenicol to inhibit bacterial growth) and then centrifuged at low speed (1,300 × *g* for 20 to 30 minutes) to remove large cell debris. The supernatant of this low speed centrifugation was assayed for infective phage and total radioactivity and will be referred to as "supernatant S" in the following. Approximately 10^{12} UV-killed T2 per ml. were added to supernatant S as carrier, a fixed amount of anti-T2 rabbit-serum, which had been exhausted repeatedly for any bacterial antibodies with a mixture of intact and broken *coli* cells, was then introduced, and the mixture incubated first for 1 or 2 hours and then refrigerated overnight (6). The precipitate which had formed was sedimented by low speed centrifugation (850 × *g* for 13 minutes), and washed three times with adsorption buffer. Radioactive counts on the precipitate were made in a windowless gas flow counter on dry samples mounted in plastic planchets.

Two groups of control experiments were carried out to test the specificity of such precipitates. In one of these, UV-killed carrier T3 bacteriophage particles (serologically unrelated to T2) were added to various supernatants S and precipitates formed by addition of strong anti-T3 serum. In the second type of control, UV-killed carrier T2 phages were added to cultures of S^{35}-labelled, uninfected bacteria which had been disrupted by sonic oscillation and precipitates formed by addition of strong anti-T2 serum. Both types of control precipitates were found to contain about 5 per cent of the total radioactivity of the mother liquor in each case. The radioactivity in these control precipitates thus represents a background of non-specific protein which is carried down together with the antigen-antibody complexes and for which a correction must be made in the calculation of the specific radioactivity of the specific precipitates from the supernatants S in the experiments reported below. It was not possible to reduce this background further either by a preliminary dialysis of supernatant S against 15 per cent mercaptoethanol (which should have prevented unspecific binding of radioactive peptides to the serum proteins through divalent metal linkages or disulfide bonds (7)), or by employing for precipitation only the γ_2-globulin fraction of the exhausted anti-T2 serum instead of the whole serum (8). The *specific* radioactivity contained in the precipitates, S_ϕ, was then estimated according to the relation

$$S_\phi = \frac{S_{ppte} - r \cdot S_{total}}{1 - r}$$

in which S_{ppte} and S_{total} represent respectively the radioactivity of the precipitates and of supernatant S and $r = 0.05$, being the fraction of the total S^{35} found to be precipitated non-specifically in the control experiments.

UV irradiations were carried out in dim light with a 15 watt Westinghouse "sterilamp" at a distance of 80 cm. During the irradiations, the suspensions of phage or infected cells were placed on a watch glass set over cracked ice (9). Incubations after UV irradiation were always carried out in dim light to minimize any possible photoreactivation.

Experimental Results

Sulfur Content of T2.—In the work reported below, the presence of phage protein has been detected by introducing radiosulfur, S^{35}, into its sulfur-containing amino acids. In order to determine the total amount of sulfur per T2 particle, the following experiment was carried out.

A number of cultures of bacteria growing in tris-glucose medium labelled with 2 to 8 mc. of S^{35} per mg. total sulfur were infected with a multiplicity of 5 T2 particles per cell and lysed by addition of M/100 KCN 100 to 200 minutes after the start of phage growth at 37°C. The lysates, which contained from 80 to 100 progeny per infected bacterium, were mixed with UV-inactivated carrier T2 particles and the phages isolated from the lysates by three cycles of alternate high-speed (10,000 *g* for 40 minutes) and low-speed (1,300 *g* for 20 minutes) centrifugations (6). The infective titer of the purified suspensions as well as the amount of S^{35} precipitable by anti-T2 serum or adsorbable to sensitive cells was assayed and from these data and from the specific activity of the growth medium the amount of sulfur per infective T2 unit calculated.

This experiment indicated that each infective T2 particle contains $2.4 \pm 0.3 \times 10^{-12}$ μg. of sulfur, in agreement with the range of values obtained by Hershey and Chase (1).

Phage Protein Synthesis in Normal Infection.—The kinetics of formation of S^{35}-labelled phage antigenic protein in bacteria infected with T2 bacteriophage was studied by breaking open the infected cells at various stages of intracellular phage development and assaying the radioactivity precipitable by anti-T2 serum by a method similar to that employed for T4 by Maaløe and Symonds (3).

A culture of bacteria was grown to a density of 2×10^8 cells/ml. in tris-glucose medium containing from 2 to 8 mc. S^{35} per mg. total sulfur, washed, resuspended in tris-buffered saline (glucose-free tris medium), and starved for 30 minutes at 37°C. (9). The culture was then infected with a multiplicity of 5 to 7 T2 phages per cell, and, after allowing 5 minutes at 37° for phage adsorption, the infected bacteria were separated from unadsorbed free phage by centrifugation, resuspended in tris-glucose medium containing the original concentration of S^{35}, and incubated at 37°C. At various times after the beginning of phage development, the infected cells were lysed by addition of M/100 KCN and (in some experiments) a high titer of UV-killed $T2r^+$ phage particles (10). These lysates were then analyzed for their content of infective phage progeny and antigenic protein.

The result of a typical experiment is presented in Fig. 1, on which the amount of T2 antigen per infected cell recovered at different times of phage development has been plotted in multiples of the amount of sulfur per T2 phage particle. It can be seen from the curve labelled "total antigen" that phage antigenic protein makes its intracellular appearance about 10 to 12

minutes after the start of phage development and then rises rapidly in amount until a level of about 75 phage units per cell has been attained by the 40th minute. The ratio of the amount of antigen to infective progeny particles is seen to be very high at the conclusion of the eclipse period and falls rapidly to a much lower value as more mature progeny particles appear. These results agree very well with the observations of Maaløe and Symonds (3) on the appearance of T4 antigen and indicate the presence of non-infective phage

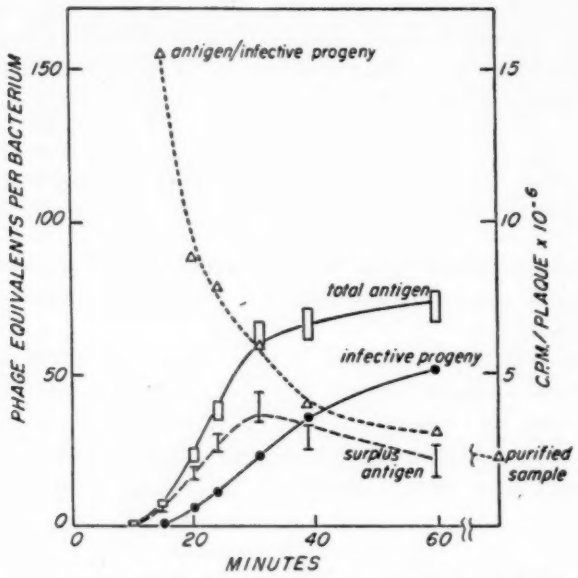

FIG. 1. Amounts of S^{35}-labelled antigen and infective progeny (left ordinate) and antigenic S^{35}-radioactivity per infective progeny (right ordinate) found in T2-infected bacteria at various times after the onset of phage development. "Purified sample" refers to the S^{35} activity per infective phage found upon isolation by differential centrifugation of the T2 particles of a late lysate of this experiment.

antigens within the infected cell. The amount of such non-infective or "surplus antigen" present at various times can be estimated by subtracting from the amount of total antigen the product of the number of infective progeny times the amount of sulfur per infective unit. The results of such calculations are also indicated in Fig. 1, where it may be seen that the surplus antigen appears to reach a constant level at 40 phage units per bacterium between 20 and 40 minutes after the onset of phage development.

As in the case of T4, these non-infective antigenic particles are lighter than the infective, mature phage particles and can be separated from the latter by differential centrifugation (3). Less than 10 per cent of such non-infective

antigens, however, were found to be adsorbable to sensitive bacteria, in contrast to the finding of Maaløe and Symonds that non-infective T4 antigen adsorbs to bacteria as readily as infective phage. In our case, non-infective protein resembles more the phage protein "doughnuts" (2), which do not adsorb to sensitive cells.

Phage Protein Synthesis after Infection with UV-Inactivated Phage.—Although there occurs neither production of infective progeny nor any synthesis of phage DNA after infection of bacteria with UV-inactivated T2, some protein still continues to be synthesized in such cells (11). In order to determine now whether the protein formed under these conditions is phage protein or

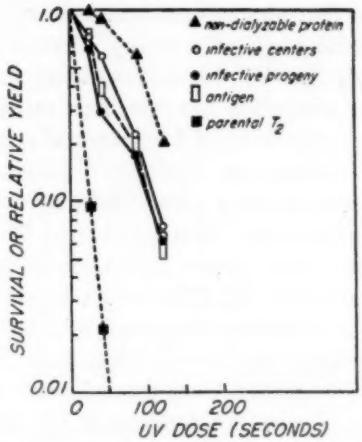

FIG. 2. Survival as infective centers and relative yield 70 minutes after the onset of phage development of infective progeny, S^{35}-labelled antigen, and S^{35}-labelled non-dialyzable protein of bacteria multiply infected with T2 particles which have received various doses of UV. The survival of the free parental T2 is also shown.

not, we have tested its precipitability with anti-T2 serum. For this purpose, non-S^{35}-labelled bacteria were infected at a multiplicity of 6 phages per cell with stocks of T2 which had received various doses of UV. The infected bacteria were then transferred to tris-glucose medium labelled with S^{35} and incubated at 37°C. for 70 minutes before being lysed by KCN. The lysates were assayed, as above, for infective progeny and for total non-dialyzable as well as antigenic S^{35}-labelled protein. The results of this experiment are presented in Fig. 2, on which the ratio of the yield of these substances formed after infection of bacteria with phage having received various doses of UV to the yield found after infection with unirradiated phage is plotted against the UV dose. Also shown in Fig. 2 is the relative survival with UV dose of the free parental T2 and of the multiply infected bacteria as infective centers when

plated before burst. (The greater resistance of the infective centers than of the parental phage to UV inactivation is due to multiplicity reactivation (12).) It is seen in Fig. 2 that the survival of the infective centers as well as the yields of infective progeny and phage antigen formed by the 70th minute after the start of phage development all decrease in the same way with the UV dose to which the parental T2 phages have been exposed before infection. It can be concluded from these results that only those UV-irradiated T2 phages and hence only those parental DNA structures can induce the synthesis of phage antigenic protein which are also able to give rise to infective progeny. The fact that the non-dialyzable protein synthesized after infection decreases more slowly with UV dose than the infective centers confirms the observation of previous workers that some protein synthesis still proceeds in bacteria infected with phages unable to give rise to any infective progeny.

Phage Protein Synthesis after UV Irradiation during the Eclipse.—The effect of UV on phage antigen production has now been examined after irradiation of T2-infected bacteria, rather than of free parental phage, at various stages of intracellular phage development. Multiply T2-infected bacteria growing in non-labelled tris-glucose medium were irradiated with different doses of UV at different times during their eclipse period and S^{35} added to the growth medium after irradiation. After further incubation at 37°C. for 60 minutes, the infected bacteria were lysed by KCN and the lysates assayed as above for the amount of infective progeny phage and S^{35}-labelled antigenic protein. The results of this experiment are shown in Fig. 3, on which the ratio of the amounts formed after UV-irradiation to those formed in the aliquot which had received no UV, are plotted as a function of UV dose. It is seen in Fig. 3, first of all, that UV irradiation of those infected bacteria in which no phage development had been allowed to proceed ($t = 0$) eliminated the formation of infective centers, phage antigen, and infective progeny in a manner similar to UV irradiation prior to infection of free T2 subsequently used to infect non-irradiated bacteria at a comparable multiplicity (*cf.* Fig. 2). At later stages of phage development, *i.e.* during the first 11 minutes after infection, the infective centers are seen to grow increasingly refractory to UV inactivation, in agreement with observations of Luria and Latarjet (13) and Benzer (9). Simultaneously, the UV sensitivity of phage antigen formation appears to decrease to the same extent. By the 16th minute after the start of phage development, when the UV sensitivity of the infective centers is seen to have increased once more, phage antigen formation has experienced a further stabilization towards UV inactivation. The relative amount of infective progeny formed, however, is seen to have experienced little change in UV sensitivity throughout this period. A striking disbalance between the amount of phage antigen and infective progeny can therefore be achieved by irradiating T2 infected bacteria at a stage later than 15 minutes after the start

of phage development. For example, the data presented in Fig. 3 indicate that the ratio *phage antigen/infective progeny* is ten times greater than normal in the aliquot irradiated for 210 seconds 16 minutes after the onset of phage development.

It is possible that the great increase in surplus antigen formed after UV irradiation at late stages of the eclipse period actually represents structurally intact progeny phages which are non-infective because they harbor non-infective DNA possessing UV lesions. If this were true, such surplus antigen should be adsorbable to sensitive bacteria, in contrast to the surplus antigen

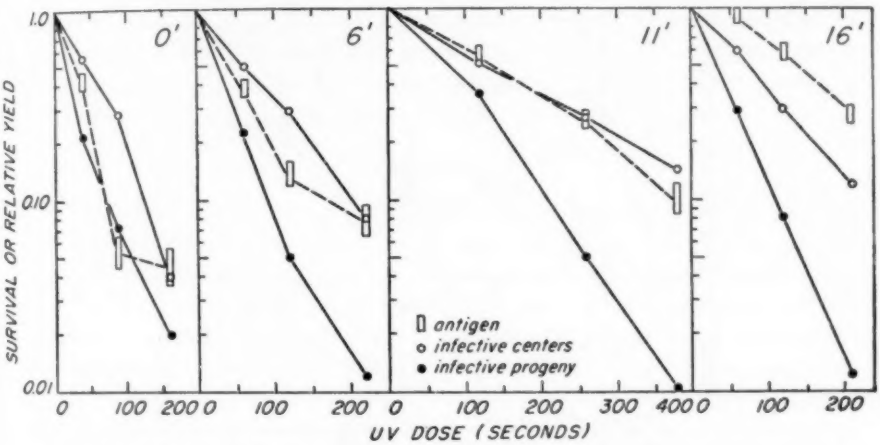

FIG. 3. Survival as infective centers and relative yield of infective progeny and S^{35}-labelled antigen 60 minutes after exposure to various doses of UV of bacteria multiply infected with T2. Each panel represents a culture in which phage development has been allowed to proceed for a certain number of minutes before UV irradiation.

formed in the course of normal infection. We have accordingly tested the adsorbability of the antigenic S^{35} in a lysate of bacteria irradiated at the 15th minute after the onset of phage growth and found that only 15 per cent of the antigenic S^{35} could attach itself to sensitive cells. The bulk of the surplus antigen in such lysates, therefore, appears to be in some form other than morphologically intact but non-infective progeny.

In order to test whether the great relative increase in surplus antigen after UV irradiation late in the eclipse reflected the possibility that once phage reproduction has proceeded beyond a certain point, the phage antigen synthesizing mechanism becomes more stable against UV irradiation than the synthesis of phage DNA, the following experiment was carried out. Bacteria were multiply infected with T2, as above, incubated in non-labelled tris-glu-

cose medium at 37°C. for 15 minutes, and irradiated with a UV dose of 150 seconds, which permitted survival of 20 per cent of the infective centers. Radiosulfur, S^{35}, or radiophosphorus, P^{32}, was then added to two different samples of the irradiated culture. The infected bacteria were incubated further at 37°C. and aliquots lysed from time to time by addition of KCN. The amounts of infective progeny and S^{35}-labelled phage antigen in the lysates were then assayed as above, while the amount of P^{32}-labelled DNA synthesized

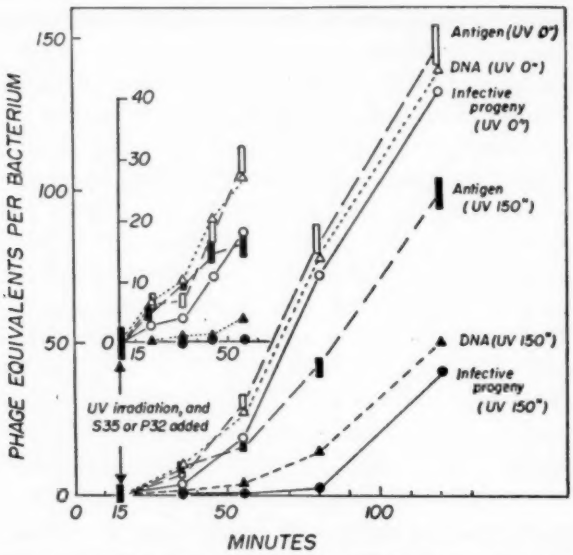

FIG. 4. Amounts of P^{32}-labelled DNA, S^{35}-labelled antigen, and infective progeny found in T2-infected bacteria at various times after the onset of phage development in a culture to which radioisotopes were added at the 15th minute. At that point, one part of the culture was UV-irradiated (UV 150") while another remained unirradiated (UV 0"). Insert presents early points with expanded ordinate.

after irradiation was determined by the extraction technique of Morse and Carter (14). Control assimilation experiments in which the same amounts of radiosulfur or radiophosphorus were added 15 minutes after infection to infected bacteria not irradiated with UV were carried out at the same time. The results of this experiment are presented in Fig. 4, where the amounts of S^{35}-labelled antigen and P^{32}-labelled DNA per infected cell have been expressed in multiples of the amount of sulfur (2.4×10^{-12} μg.) and phosphorus (2.3×10^{-11} μg.) (15) per infective T2 particle. It is seen, first of all, that in the non-irradiated control, the amounts of infective progeny, phage antigen, and DNA formed subsequently to 15 minutes increase hand in hand with each other. In the UV-irradiated culture, on the other hand, formation of infective progeny as

well as of DNA is almost completely arrested for some 60 minutes after irradiation, after which time their synthesis resumes. Phage antigen, however, continues to be formed at almost a normal rate for the first 20 to 30 minutes after UV irradiation, after which time it proceeds at a somewhat reduced rate.

Thus it is evident that by the 15th minute after the onset of phage development the reactions necessary for phage protein production have indeed become more refractory to inactivation by UV than the processes of phage DNA synthesis. After that time, furthermore, there appears to be no direct relation between the rates of phage DNA and phage protein synthesis, since protein synthesis can take place at an almost normal rate while DNA synthesis is arrested. This finding complements previous observations that at this same late stage of the eclipse period phage DNA synthesis can proceed after phage protein synthesis has been suppressed by addition of inhibitors or removal of required amino acids (16–18).

DISCUSSION

The present experiments show that at the outset of T2 phage infection the two functions of the DNA of the parental phage, *i.e.* causing its own reduplication and inducing the synthesis of phage protein, are equally sensitive to inactivation by UV, suggesting that the integrity of quantitatively equivalent parts of the phage DNA is required for the successful initiation of both reactions. After the synthesis of phage-specific protein has proceeded to the point at which the first phage antigen makes its intracellular appearance (*cf.* Fig. 1), the protein-synthesizing mechanism has become very much more resistant to UV irradiation than the DNA-synthesizing mechanism, so that phage antigen can continue to be formed at a high rate even after UV irradiation at some late stage in phage development has abolished further DNA synthesis (*cf.* Fig. 4). Two possibilities can be considered for the reason why during the eclipse period phage antigen formation becomes more resistant to UV irradiation than DNA synthesis, depending on whether one envisions a *direct* or an *indirect* control of protein formation by the phage DNA throughout the eclipse period. If the control is *direct*, then there may occur a change of state or structure of the phage DNA at the onset of its replication during the eclipse period, so that UV lesions on the vegetative DNA do not interfere as much with its protein- synthesizing functions as with its self-duplication. If the control is only *indirect* then there may occur a transfer of the "information" pertaining to the specificity of the phage antigen from the parental DNA to another substance which is more resistant to UV irradiation than DNA, *e.g.* ribonucleic acid, protein, or ribonucleoprotein, and which can still direct the synthesis of antigenic protein after UV irradiation has destroyed the parental DNA. Although it is difficult to decide between the two alternatives on the basis of the available evidence, the recent

demonstration of the synthesis of an essential, non-phage-antigenic protein (16–18) and of a special ribonucleic acid (19) at the onset of T2 and T4 reproduction is in good agreement with the second hypothesis of *indirect control* of the synthesis of the antigenic phage protein by the DNA of the infecting phage.

SUMMARY

The amount of phage-specific protein in T2-infected bacteria growing in a medium containing radiosulfur, S^{35}, has been studied by measuring the radioactivity in specific antiphage serum precipitates of lysates. In the course of normal infection, non-infective phage antigen has been found to make its first intracellular appearance shortly before the end of the eclipse period, in agreement with the findings of Maaløe and Symonds with phage T4. No such phage antigen is produced either in bacteria infected with UV-inactivated T2 or in T2-infected bacteria whose survival as an infective center has been destroyed by UV irradiation during the early stages of the eclipse period. If the infected bacteria are UV-irradiated only at later stages of the eclipse period however, then phage antigenic protein continues to be synthesized in those infected cells in which DNA synthesis and, *a fortiori*, production of infective progeny have been almost completely suppressed. It is concluded from these results that once the mechanism for formation of phage-specific protein has been established within the infected cell under the influence of the parental DNA, synthesis of phage-specific protein can continue independently of the synthesis of phage DNA. The possibility that the phage DNA controls the specificity of the phage protein indirectly through substances other than DNA is discussed.

The author is greatly indebted to Dr. Gunther S. Stent for his valuable suggestions and criticisms.

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CHEMICAL TRANSMISSION AND ADAPTATION

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(Received for publication, September 4, 1956)

INTRODUCTION

The discovery of the stretch receptors of crustaceans (1, 2) and the recognition of their function by physiologists (3–6, 8, 12, 16) have opened new possibilities for the attack on neurophysiological problems of general interest. One becomes more and more convinced that the sensory neurons of these receptors can be considered as models of neurons in general. There is evidence that these neurons receive an efferent regulatory innervation and thus it becomes feasible to study general problems of synaptic transmission in stretch receptor preparations.

According to histological studies (9) the stretch receptor neurons of the crayfish receive endings of at least one efferent fiber and there is a possibility that they are also innervated by a second fiber, just as is the case in the stretch receptors of lobsters (1). The findings of Kuffler and Eyzaguirre (13) show conclusively that there is one efferent fiber which inhibits the discharge of the sensory stretch receptor neurons of the crayfish. If there is another efferent supply this might be excitatory (1, 9, 13).

Wiersma, Furshpan, and Florey (16) have described the excitatory effect of acetylcholine in these receptors and its potentiation by eserine as well as its block by atropine. Further pharmacological studies have shown that the receptor neurons are specifically sensitive to acetylcholine and one is tempted to assume that acetylcholine is the transmitter substance of a regulatory excitatory fiber as is the case in the heart ganglia of the crayfish (17). Actually this assumption is based on the following points: (*a*) The rather specific effect of acetylcholine (5) points to the existence of a specific acetylcholine receptor substance on the neuron. (*b*) The potentiating effect of eserine indicates the presence of choline esterase which is usually connected with cholinergic transmission. (*c*) Atropine does not block the response of the neurons to stretch but blocks applied acetylcholine (16). It would therefore seem unlikely that acetylcholine is involved in the transmission of excitation from stretched muscle to neuron. It is, however, quite likely that acetyl-

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choline is normally involved in the transmission of excitation from regulatory nerve endings to the sensory neuron (a view already expressed by Wiersma, Furshpan, and Florey (16)).

It has been shown that the stretch receptor neurons also respond to an inhibitory substance, factor I (5). There are strong arguments in favor of the view that the active principle of factor I is a transmitter substance of inhibitory neurons in crustaceans as well as in mammals (5, 7, 10, 11). In the stretch receptor neurons factor I blocks or diminishes the response to stretch and counteracts the action of acetylcholine.

So far we feel justified in assuming that acetylcholine and factor I are the transmitter substances of regulatory fibers whose endings make synaptic contact with the dendrites (and cell body?) of the stretch receptor neurons (9).

The response of the "slow adapting" neuron of crayfish stretch receptors to sudden increase or decrease of stretch has been described in a recent publication (8). It could be shown that a sudden increase in the length of the receptor muscle fibers is followed by an immediate jump in the impulse frequency which is followed by adaptation, consisting of a gradual decline of frequency until asymptotically a steady state is reached. Sudden relaxation of the muscle is accompanied by an immediate fall in discharge frequency (silent period) followed by a gradual return of impulses until a steady state frequency is reached. Adaptation was thus shown to take place in two directions and has been considered as an active process that leads to a return of the displaced membrane potential to a level close to the previous resting potential (which itself was the result of adaptation!). Adaptation can actually be directly compared with the well known process of accommodation in nerve fibers.

Method

Crayfish of the species *Cambarus virilis* Hagen were used as experimental animals. The stretch receptors were dissected and set up in the manner described previously (5). The dorsal half-shell of the "tail" served as a receptacle for the test solutions. It was freed from muscles except for the most lateral strand of the musculus superficialis lateralis (Schmidt (14)) which was left in place in that segment in which the stretch receptor organ was prepared. The nerve trunk containing the stretch receptor neurons was left attached to this muscle (Fig. 1). It was thus possible to prevent the nerve trunk from pulling on the delicate sensory nerve cells and their endings when the fluid was changed. Preparations in which changing of the fluid induced changes in the impulse frequency of the receptor neurons, were discarded.

Van Harreveld's salt solution (15) was used as saline medium. The addition of sodium-bicarbonate was, however, omitted. This solution will be called "crayfish solution." Factor I has been prepared according to the methods described by Florey and McLennan (10) and Elliott and Florey (3), but fresh brain extracts were also used repeatedly. The latter were prepared by boiling and homogenizing fresh or frozen

beef or rat brain in a tenfold (weight) amount of K-free crayfish solution. Acetylcholine as well as purified factor I was dissolved in crayfish solution.

The activity of factor I and its dilutions will be expressed in crayfish units (C.U. (3)), the crayfish unit being defined as the amount of factor I/ml. which is able to block the discharge of the slow adapting stretch receptor neuron of the third abdominal segment of a specimen of the crayfish *Cambarus virilis* Hagen for 10 seconds. Since variations of the sensitivity of stretch receptor neurons to factor I occur, a standard preparation of factor I was assigned the activity of 100 crayfish units per ml. (accord-

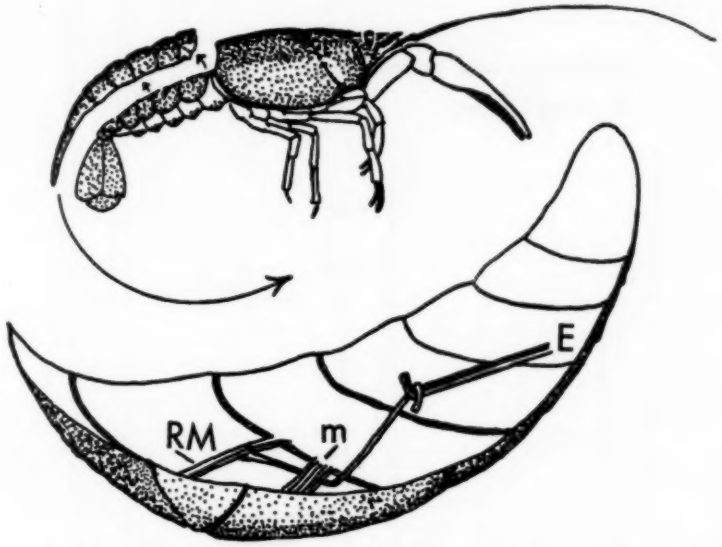

FIG. 1. This diagram represents the stretch receptor preparation as used in the adaptation experiments. *RM*, the stretch receptor muscles with the sensory neurons attached; *M*, musculus superficialis lateralis which remains in the segment in order to keep the nerve trunk anchored. *E*, platinum electrode over which the nerve is laid.

ing to the average sensitivity of a great number of test preparations). Determinations of activity on individual test organs were corrected for deviations in the sensitivity of the particular stretch receptor preparation to the standard solution of factor I. The corrected (absolute) values for factor I were thus expressed in "crayfish units reference" (C.U.R. (3)). In previous publications (7, 10, 11) the activity of factor I has been expressed in brain equivalents (B.E.).

In all the experiments to be mentioned in this paper the fast adapting nerve cells of the stretch receptor preparations were either crushed with the tips of fine forceps or their impulses, if they appeared at all, were disregarded. The nerve which contains the sensory axons was hung over a thin platinum electrode. Nerve impulses were recorded with an A.C. preamplifier and a single beam oscilloscope. An electronic counter together with a timing unit was used to determine the impulse frequencies at given intervals:

Usually three second counts were used, at intervals of 0, 15, 30, 60, and 90 seconds after application of saline or "transmitter"-solution.

RESULTS

(*a*) *Acetylcholine.*—The stretch receptor neurons of *Cambarus virilis* Hagen are not quite so sensitive to acetylcholine as are those of *Cambarus clarkii* Girard (16). However, concentrations down to 1 μg/ml. are usually effective

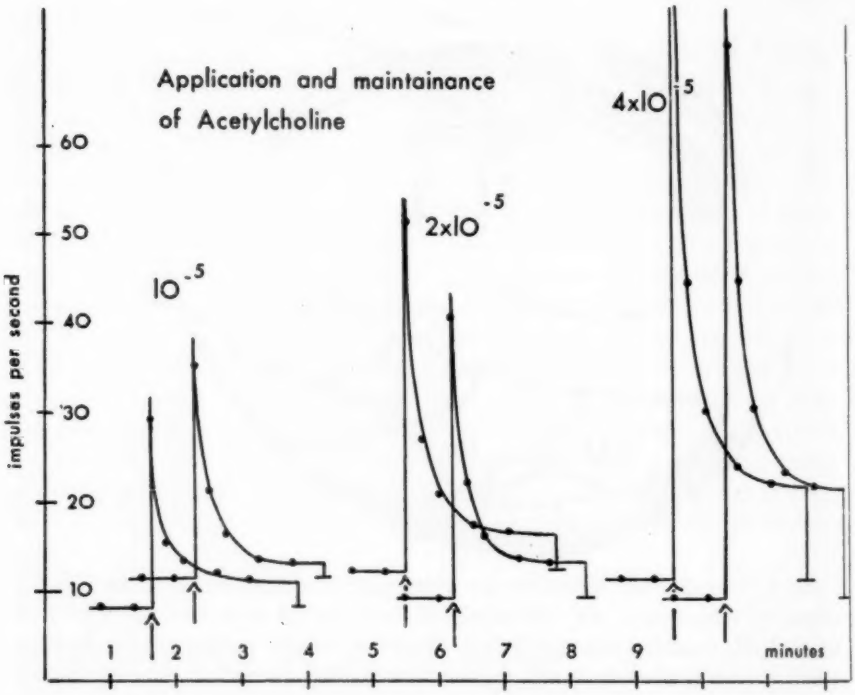

FIG. 2. Response of one slow adapting stretch receptor neuron to applied and maintained acetylcholine of various concentrations. The drug was applied at the arrows and the vertical lines at the end of each sample curve represent the increase of the steady state frequency over the original frequency level.

in producing a marked increase in the impulse frequency of the sensory neuron. This excitation induced by acetylcholine follows a characteristic pattern: immediately after the application of acetylcholine the frequency jumps to a peak value whereupon it declines again, rapidly at first and then more slowly until a steady state is reached. Fig. 2 gives typical examples of several measurements taken from the same nerve cell. The values show a certain variation, but the pattern is obvious: after a sudden rise the frequency declines,

following an exponential curve which asymptotically approaches a steady state value. As shown in this figure, the sudden rise as well as the final steady state is proportional to the concentration of acetylcholine applied. There

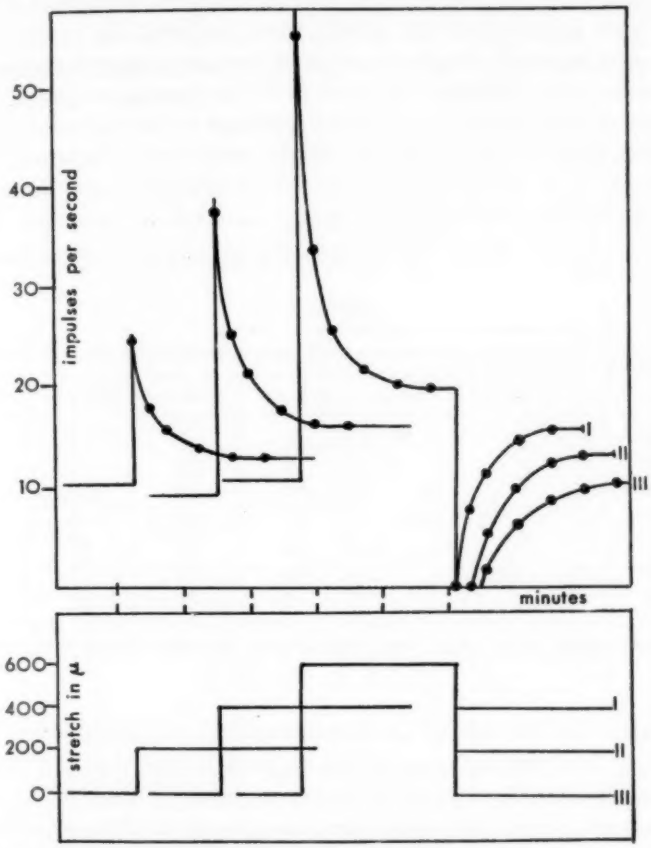

FIG. 3. Responses of one slow adapting stretch receptor neuron to sudden increases and decreases in stretch of the receptor muscles. Note the difference in adaptation time with different amounts of relaxation of the muscles. The muscles were stretched by bending the abdominal half shell. The changes of length of the receptor muscles were estimated, using a micrometer eyepiece on the dissecting microscope.

is, however, no marked difference in the time required to reach the steady state (the "adaptation time") with different concentrations of acetylcholine.

The response of the sensory neuron to acetylcholine is obviously similar to the response to an increase in stretch and there seems to be no objection to the use in both cases of the term *adaptation* with respect to the fact that the maximum response is followed by a returning of the impulse frequency

to a level which is closer to the original frequency. To illustrate this statement Fig. 3 gives examples of measurements taken from the same nerve cell as the one used in Fig. 2, during stretch experiments. A more detailed analysis of the response of stretch receptor nerve cells to sudden changes in the length of the receptor muscle fibers has already been published (8). In this previous study it could be shown that the amount of increase of stretch does not alter the adaptation time although the amount of the immediate rise of the frequency and the final steady state are proportional to the increased stretch.

Since the adaptation of the sensory neuron follows an asymptotic curve no definite endpoint of the adaptation can be determined unless we specify a certain minimum rate of frequency change as arbitrary endpoint. For our purposes a minimum change of 0.25 per cent/sec. seems to be adequate. We

TABLE I

Concentration of acetylcholine applied	No. of cases in which a steady state of the impulse frequency was reached in					
	15–30 sec.	30–60 sec.	60–90 sec.	90–120 sec.	120–150 sec.	150–180 sec.
10^{-6}	11	23	26	17	3	
5×10^{-6}		2	18	36	8	
10^{-5}			11	48	7	
2×10^{-5}			12	62	9	
4×10^{-5}			13	65	11	2
6×10^{-5}			13	71	15	1
10^{-4}			4	75	25	3

The values presented in this table were obtained from seventeen stretch receptor preparations.

can now state that the adaptation is accomplished in approximately 2 minutes, whether the preceding stimulus was an increase in stretch or the application of acetylcholine. Representative values are given in Table I.

If the acetylcholine is now withdrawn and replaced by plain saline medium, the frequency decreases instantly to a lower level but immediately it begins to rise again, fast at first and then more slowly until it reaches the original value, that is the impulse frequency before the application of acetylcholine. The length of the silent period which follows the withdrawal of acetylcholine is proportional to the amount of acetylcholine that had been applied. Fig. 4 gives several examples. It becomes obvious that the adaptation time here *depends* on the concentration of acetylcholine removed.

Again, the situation is strikingly similar to that of a stretch receptor preparation whose muscle fibers are relaxed by varying amounts (see Fig. 3). In both cases the adaptation time increases with the amount of stimulus (stretch or acetylcholine) removed.

It is now interesting to compare the effect of acetylcholine on a stretch

receptor preparation whose muscle fibers were somewhat more stretched so that the steady state impulse frequency was raised. Although the variability of the responses was great it can be said that a preparation with a high rate of firing responds to acetylcholine by giving about the same number of additional impulses per second as a "low frequency" preparation. Table II gives representative values. As can be seen from this table the effect of acetylcholine is strictly additive.

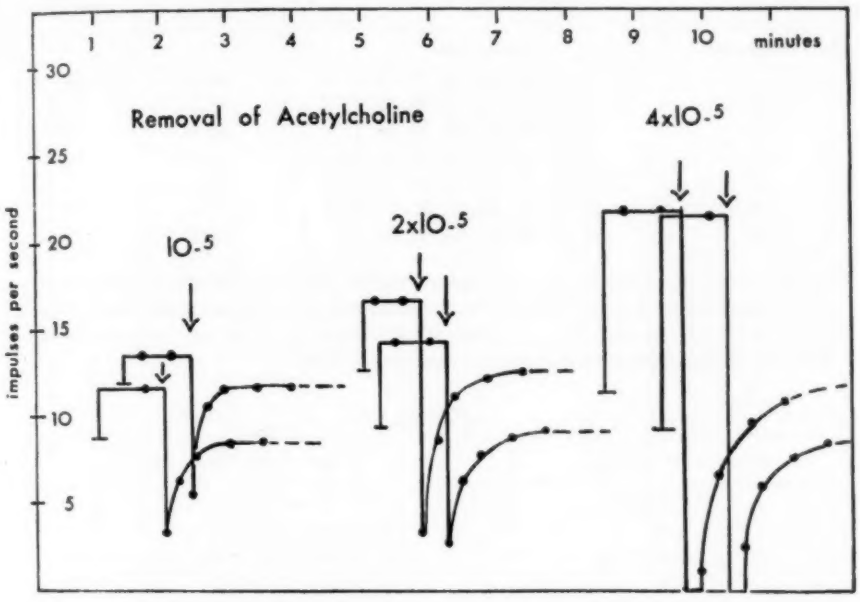

FIG. 4. Inhibition induced by removal of various concentrations of acetylcholine (see Fig. 2). The drug was removed and replaced by plain saline medium at the arrows. The vertical lines at the beginning of each curve represent the increase of the steady state frequency previously effected by acetylcholine. Note the differences in adaptation time.

If acetylcholine was removed from a preparation with a high impulse frequency the silent period was shorter than if it was removed from a preparation that was firing at a low rate. The difference, however, becomes obvious only if the original impulse frequencies approach the extremes of the lowest or highest steady state values. Medium differences in the frequency level seem to be without influence on the silent period.

(*b*) *Factor I.*—The response of a stretch receptor neuron to a solution of factor I is in every detail the opposite of that to a solution of acetylcholine. There is an immediate drop in frequency but if the concentration of factor I

is low enough, the impulse frequency rises again until it reaches asymptotically a steady state value which is somewhat lower than the original frequency level. Concentrations of factor I higher than 0.5 C.U.R./ml. usually cause a silent period whose duration is determined by the concentration of factor I

TABLE II

Original frequency	Steady state frequencies after application of acetylcholine 2×10^{-4}	Average	Average increase due to acetylcholine
impulses/sec.	*impulses/sec.*		
5	14, 14, 15, 15, 16, 17, 18	15.5	10.5
10	14, 19, 21, 22, 22, 25, 26	21.2	11.2
15	24, 25, 25, 27, 29, 29, 29	26.8	11.8
20	30, 30, 30, 32, 32, 34, 34, 36	32.2	12.2
25	30, 35, 35, 35, 37, 41, 41, 41	36.4	11.4
35	42, 43, 45, 46, 49, 50, 51, 55	47.6	12.6
50	58, 58, 59, 62, 66, 66, 67	62.2	12.2

The values presented in this table were obtained from one individual neuron, using repeated application of acetylcholine. The "original frequency" is the steady state frequency after the neuron has adapted to the normal saline medium. It has been adjusted to various levels by changing the tension of the receptor muscle fibers.

TABLE III

Concentration of factor I	Length of silent period, *sec.*
C.U./ml.	
0.6	0, 0, 0, 0, 0, 1, 1, 2, 2
0.8	1, 1, 2, 2, 2, 2, 3, 3, 4
0.9	2, 4, 4, 4, 5, 6, 6, 6, 7
1.0	8, 8, 10, 10, 10, 10, 11, 11, 12
1.1	15, 15, 15, 16, 16, 17, 18, 18, 18
1.2	25, 26, 27, 27, 29, 35, 36, 37, 60
1.3	29, 39, 46, 48, 65, 77, 120, 120, 120

The values presented in this table were obtained from one individual stretch receptor neuron. The sequence of the different concentrations applied was varied in order to exclude effects of possible changes in sensitivity of the neuron as far as possible. The maximal time of contact between the neuron and factor 1 solutions was 120 seconds.

as well as by the sensitivity of the receptors to this factor. Table III gives representative values for the correlation between concentration of factor I and length of silent period.

Immediately after the replacement of the factor I solution by plain crayfish solution the frequency jumps to a peak value far above the original frequency and then decreases at once to a steady state level which is more or less equal to the steady state frequency before the application of factor I.

This effect can be seen in Fig. 5. Thus the removal of factor I has an effect similar to that of application of acetylcholine or of a sudden increase in stretch.

If the tension of the receptor muscle fibers was increased so that the resulting steady state frequency was about 20/sec., the silent periods were shorter and the course of adaptation was more rapid than in less stretched prepara-

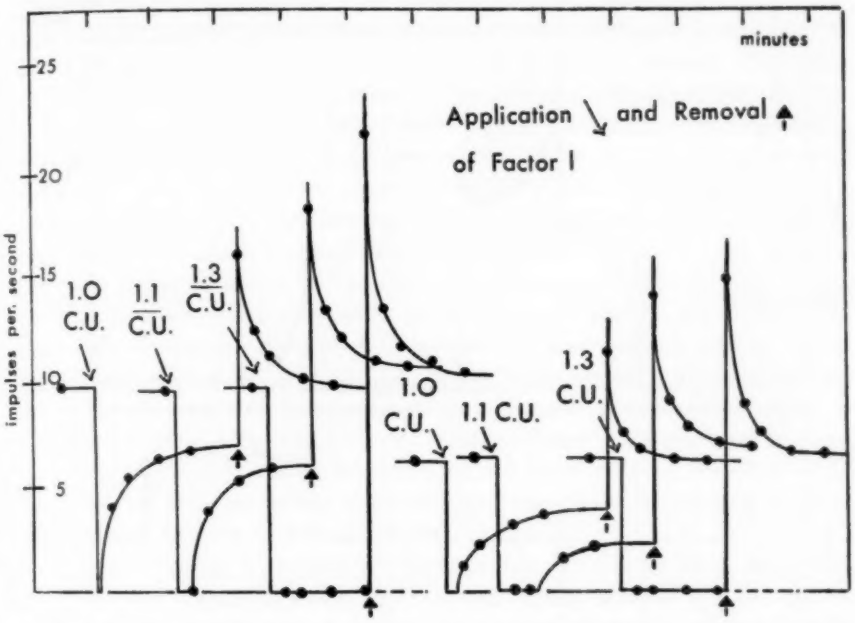

FIG. 5. Responses of one slow adapting stretch receptor neuron to application and removal of various concentrations (expressed in C.U. = crayfish units (per ml.)) of factor I.

tions with frequencies of about 5 impulses per second. The excitatory effect of removal of factor I is, however, quantitatively the same whether it occurs in a stretched or a relaxed preparation. It is purely additive and follows the same time course regardless of the frequency level of the nerve cell and depends only on the concentration of factor I removed.

(*c*) *Stretch Responses in the Presence of "Transmitters."*—If during the excitatory action of acetylcholine the tension of the receptor muscles is lessened, there occurs a sudden fall in the frequency which is followed by a return to a steady state frequency which is lower than that originally induced by acetylcholine. If during the action of acetylcholine the muscles of the receptor organ are stretched, there occurs a sharp rise in frequency and an

asymptotic return to a steady state level somewhat above the acetylcholine-induced frequency.

The opposite is true for factor I. In its presence the preparation behaves as if relaxed. If the concentration of factor I is sufficiently low (*e.g.* 1.5 C.U.) but still blocking the discharge indefinitely, sudden increase in the stretch of the muscle fibers will cause the appearance of nerve impulses of moderate

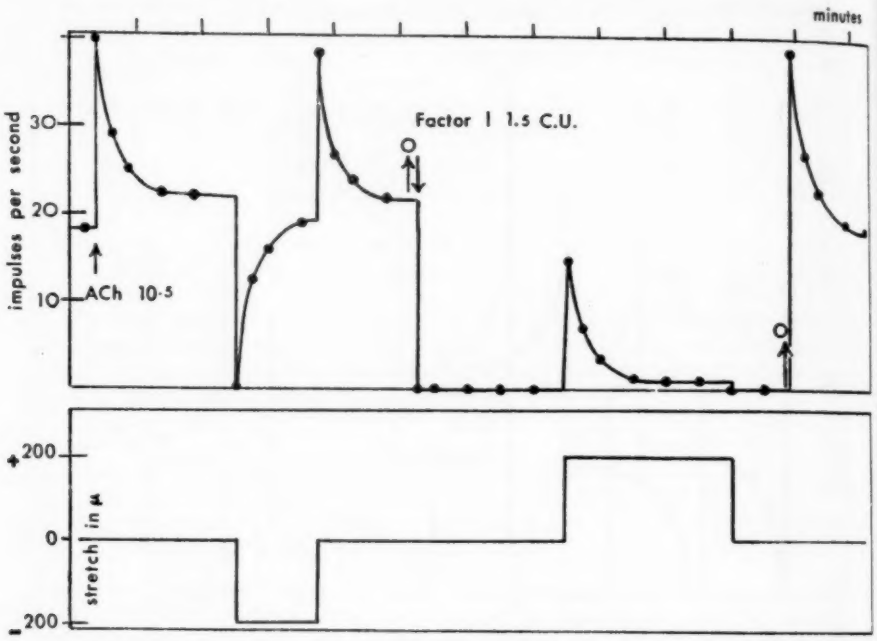

FIG. 6. Responses of a slow adapting stretch receptor neuron to stretch or relaxation of its muscle fibers while treated with acetylcholine or factor I. 0, washing with saline medium.

frequency or even high frequency, but at once this frequency will decline and return to zero or a low frequency which then is determined by the interaction of stretch and factor I.

Examples of this behavior are shown in Fig. 6.

DISCUSSION

The present as well as previous (8) findings demonstrate that receptor neurons adapt not only to excitatory but also to inhibitory stimulation and that these neurons respond in this way not only to the stimulus (whatever its nature may be) but also to the removal of the stimulus. Removal of an excitatory stimulus to which a nerve cell has adapted, causes the same response as

an inhibitory stimulus and removal of the inhibitory stimulus is followed by a response identical with that of an excitatory stimulation.

This behavior cannot be found if the stimulus acts only briefly. It has been shown (8) that if the receptor muscle fibers are suddenly stretched and immediately relaxed to the original tension, the impulse frequency instantly rises to a high value (corresponding to the stretch applied) but returns without delay to its original level as the tension decreases; *there is no inhibition of the discharge.* Only if the increased stretch is maintained sufficiently long to permit adaptation will inhibition occur as the stretch is released. The same (with reversed signs) is true for sudden relaxation and immediate restretching. The adaptation process thus has a much greater inertia than the immediate response of the neuron. Frequency changes elicited by excitatory or inhibitory stimulation (whatever the nature of the stimulus may be) are therefore the resultant of the differential between the inertia of the immediate response and that of adaptation (even if adaptation were understood only as the exponential factor of decay of the immediate response). This in turn means that the response of the neuron to excitatory or inhibitory stimulation will depend on the rate of change of those environmental factors which, due to their changing, provide the stimulus. In the extreme case, that is when the rate of change equals the time course of the adaptation process, the immediate response should be identical with the "steady state." This situation is comparable to the *Einschleich* phenomenon of the nerve fiber, in which there is no conducted response if the time course of the rising stimulating current equals the time course of accommodation.

It has been noted on several occasions in this and the previous paper (8) that the adaptation time seems to be rather independent of the size of the stimulus if this is excitatory, but that it is proportional to the stimulus if the stimulus is inhibitory in nature. Obviously there must be two different mechanisms at work. This is the more probable as it seems logical to assume that an increase in the discharge rate is caused by a different factor than the slowing of this rate.

We know that stretch depolarizes nerve endings and cell body of the stretch receptor neuron and that the frequency of firing of the neuron depends (within limits) on the level of the membrane potential. This has already been extensively discussed (6, 8, 12, 13). Acetylcholine is known to be a depolarizing substance and we may assume that factor I has repolarizing properties.[1] The impulse frequency could then be taken as a measure of the membrane potential. Adaptation, as measured by the impulse frequency, would thus also be a phenomenon of the membrane potential. The processes involved in

[1] This is presently being studied by means of intracellular microelectrodes, in collaboration with Dr. Robert Martin.

adaptation might therefore be those which are involved in the maintenance and change of the membrane potential of nerve cells. It seems now to be of considerable interest to study the effect of variations in the ionic composition of the external saline medium on adaptation.

Whatever the mechanism of adaptation may be, it seems important that a neuron has been found to adapt to the action of those chemicals which are considered to be transmitter substances normally active on this nerve cell. This means that a neuron is able to shut itself off from the action of a released transmitter, even if this transmitter is not removed by circulation or enzymatic action. It seems interesting to speculate along the line that even the neurons in the central nervous system or in autonomic ganglia can be considered as "sensory" or receptor neurons inasmuch as they respond to chemical stimuli, that is to transmitter substances. We should therefore expect to find adaptation also taking place in central neurons. Phenomena such as post-excitatory depression or postinhibitory excitation would thus find an obvious explanation.

The findings reported in this paper perhaps open a new perspective for the study of drug actions: it is very well conceivable that neurogenic drugs act not only by replacing or blocking transmitter substances, inhibiting antitransmitter enzymes or directly affecting membrane potentials, but they might very well affect the mechanism of the adaptation process. A study of the action of drugs on the time course of adaptation seems, from this point of view, promising.

SUMMARY

Acetylcholine and factor I appear to be transmitter substances of excitatory and inhibitory regulatory nerve fibers supplying the sensory neurons of stretch receptor organs of the crayfish.

Sudden application of a low concentration of acetylcholine causes the impulse frequency to jump to a peak value. But immediately the frequency falls again and gradually reaches a steady state which is not far above the previous frequency level. If the acetylcholine is now withdrawn there follows a silent period after which the frequency returns to its original level. The time course of these events is identical with that of adaptations to sudden increase or decrease of stretch.

Factor I in sufficiently low concentrations causes an immediate fall in impulse frequency (silent period) which is followed by a return to a value near the previous frequency level. Withdrawal of factor I is followed by excitation and again return of the frequency to the rate measured before the application of factor I. The time course of these phenomena is identical with that of adaptations to sudden decrease and increase of stretch.

It is suggested that adaptation may be a property not only of sensory

neurons but of neurons in general and that even central neurons may be considered as receptor neurons inasmuch as they respond to chemically transmitted excitatory and inhibitory stimuli.

The author wishes to express his gratitude to Dr. K. A. C. Elliott for his continuous and stimulating interest in this work and to thank him as well as Dr. H. H. Jasper and Dr. Robert Martin for their encouragement, advice, and criticism.

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ADAPTATION OF BACILLUS MEGATHERIUM TO TERRAMYCIN (OXYTETRACYCLINE)

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(Received for publication, September 10, 1956)

SUMMARY

B. megatherium cultures contain a few cells per hundred million which are able to grow in the presence of terramycin. Growth of the other cells is inhibited by the antibiotic with the result that the resistant cells overgrow the culture. The resistant cells are present in the culture, without contact with terramycin, and are able to grow in the presence of terramycin, without further modification.

The resistant cells are mutants of sensitive cells.

Adaptation of B. megatherium to Terramycin

The addition of low concentrations (0.01 to 0.1 γ/ml.) of terramycin to *B. megatherium* cultures in liquid media results in a slight decrease in the growth rate of the culture, but does not cause a lag period. On solid media the colonies grow more slowly, but the number of colonies remains the same. No adaptation results in either liquid or solid media.

Higher concentrations (0.1 to 5 γ/ml.) cause a decrease in the growth rate in liquid cultures and a great decrease in the number of colonies on solid media. The overgrowth in liquid media and the surviving colonies on solid media are resistant to much higher concentrations of terramycin than is the original culture.

The resistant colonies come from the few resistant cells present in the original culture. This resistant strain may be isolated by Lederberg's replica technique and therefore does not arise as a result of adaptation of sensitive cells to the antibiotic. The resistant strain isolated by the replica technique is indistinguishable from a strain derived from colonies surviving exposure to terramycin. The resistant cells in the culture,[1] therefore, are capable of growing in the presence of the antibiotic without further modification.

[1] Resistant cells are referred to for the sake of simplicity as though there were only one kind of resistant cell, or mutant. Actually, there is undoubtedly a whole series of

The resistant cells arise from sensitive cells, since a few thousand cells of the sensitive culture when plated on agar containing terramycin, form no colonies, while cells from the same suspension when plated on YEP agar form colonies and every colony contains resistant cells. These resistant cells must have come from sensitive cells.

The ratio of resistant to sensitive cells rises sharply at first and then remains constant. The constant ratio varies from $1/10^8$ to $1/10^5$ depending on the strain of *B. megatherium*, and the culture medium.

Prolonged growth of the culture in the steady state apparatus in the presence of increasing concentrations of terramycin results in a strain capable of growth in the presence of 200 γ terramycin/ml. (about 1000 times the original inhibiting concentration). No further adaptation could be obtained.

All adapted strains revert sooner or later to the sensitive strain, depending on the culture medium. This reversal probably is due to overgrowth by a sensitive mutant, since it can be caused to take place by the addition of sensitive cells to the resistant strain.

The results in general are similar to those described by Fildes and Whitaker (1948), Finlay *et al.* (1950), Bryson and Demerec (1950), Hobby and Lenert (1950), Cavalli (1952), Welsch (1952), and others (*cf.* Third Symposium of Society of General Microbiologists).

Experimental Results

The effect of various terramycin concentrations on the lag period, growth rate, maximum cell concentration, and colony count is shown in Table I. As the terramycin concentration increases from 0 to 0.5 γ terramycin/ml., the growth rate decreases, but no lag occurs. The culture does not become resistant.[2] Increasing the terramycin concentration from 0.5 to 5 γ/ml. results in an increase in lag, a decrease in growth rate, and a sudden decrease in colony count to about $20/10^8$ cells. The overgrowth in this range of terramycin concentration is resistant. Higher concentrations prevent growth and do not result in resistant cultures. Concentrations over 50 γ/ml. kill the cells. The results in general confirm those of Hobby and Lenert (1950).

These results indicate that the emergence of a resistant from a sensitive culture is the result of the overgrowth of the sensitive cells by a few resistant cells. The effect of terramycin on the growth rate of sensitive and resistant cells confirms this (Fig. 1). It is evident that the resistant cells grow at about the same rate in the presence or absence of terramycin, whereas the sensitive cells grow much more slowly in the presence of the antibiotic. The result is that, in the

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mutants which differ from each other with respect to their growth rates in the presence of the antibiotic. For the present purposes, the term TR mutants refers to the group which are able to grow in the presence of >2, < 5 γ terramycin/ml.

[2] Prolonged growth in 0.2 γ terramycin/ml. does result in resistant strains.

presence of the antibiotic, the resistant cells overgrow the culture, while in the absence of terramycin, the proportion of resistant/sensitive cells remains the same.

These results prove that some cells in the culture are able to grow in the presence of the antibiotic, but do not indicate whether the resistant cells arise as a result of the adaptation of a few sensitive cells to the antibiotic or are present in the culture before the antibiotic is added.

TABLE I

Effect of Terramycin Concentration on Growth Rate and Development of Resistance in B. megatherium

899*a* in log growth diluted to 5×10^6 B/ml. in YEP containing terramycin noted. 10 ml in test tubes. Shaken 35°. B/ml. determined by turbidity. After 30 hours, culture plated on YEP agar containing terramycin noted.

Terramycin/ml., γ.....	0	0.2	0.5	1.0	2.0	5.0	10.0	20	50
Lag, *hrs*.....	0	0	0	10	20	24	>24	>24	Indefinite
K_g-hr.$^{-1}$ (at first).....	1.8	1.6	0.9	0	0	0	0	0	0
B/ml. after 30 hrs.....	1×10^9	1×10^9	1×10^9	1×10^9	6×10^8	5×10^7	5×10^6	5×10^6	5×10^6

Overgrowth from Above Tubes Plated on Agar + Terramycin Noted Below

Terramycin/ml.	Colonies/ml.								
γ									
0.2	$>10^7$	$>10^7$	$>10^7$	$>10^7$	$>10^7$	$>10^7$	$>10^6$	$>10^6$	0
0.5	50	46	40	$>10^7$	$>10^7$	$>10^7$	40	60	
1.0	30	25	32	$>10^7$	$>10^7$	$>10^7$	34	45	
2.0	14	20	24	$>10^7$	$>10^7$	$>10^7$	30	22	
5.0	18	20	16	$>10^7$	$>10^7$	$>10^7$	22	30	
10.0	0	0	0				0	0	

The resistant strains may be isolated by the Lederberg (Lederberg and Lederberg, 1952) replica technique (Table II), however, and therefore are present in the culture and do not arise as the result of the adaptation of the sensitive cells to the antibiotic.

It is still possible to assume that the resistant cells change after contact with the terramycin before they are capable of growth in the presence of the antibiotic.

The lag period, growth rate, and colony count of a sensitive strain, a resistant strain isolated from the overgrowth in 3 γ terramycin/ml., and a resistant strain isolated by the replica technique were, therefore, determined in the pres-

ence of various concentrations of terramycin (Table III, Fig. 2). The replica resistant strain had never been in contact with terramycin until the start of the experiment and, therefore, would be expected to show a lag period or a slightly lower growth rate, if any adaptive changes occurred before the cells

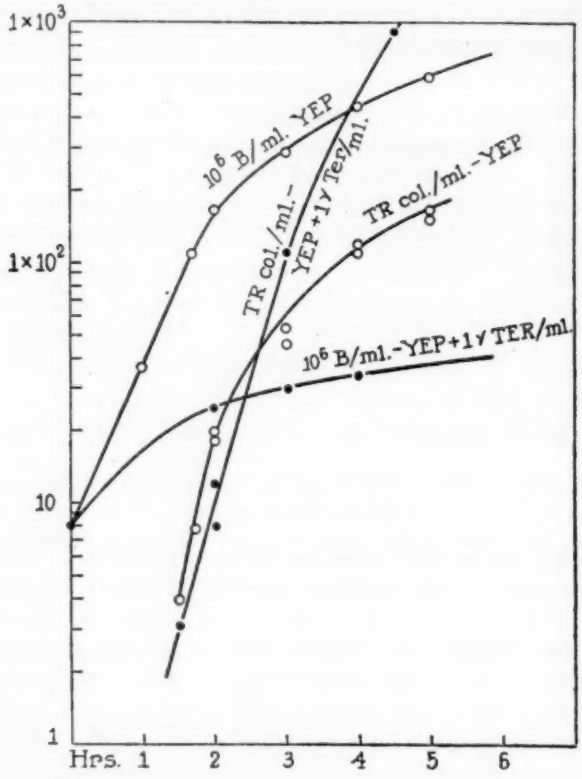

FIG. 1. Growth rate of total cells/milliliter and of TR cells/milliliter in YEP and YEP + 1 γ terramycin/ml. Cultures shaken at 35°.

were able to grow in the presence of the antibiotic. This is not the case. The replica strain, when first exposed to the antibiotic, is indistinguishable from the overgrowth strain which had grown many generations in the presence of the antibiotic. There is no indication, therefore, of any adaptive change in the cell as a result of growth in the presence of terramycin. This conclusion is confirmed by the fact that the sensitive strain may be grown in the presence of lower concentrations of terramycin (0 to 0.1 γ/ml.) indefinitely without any adaptation. In this range the growth rate of the cells is not affected. As soon as

TABLE II

Isolation of Terramycin Resistant B. megatherium from Stock 899a Culture by Replica Technique

	Plate No.	Terramycin /ml. agar	Colonies/plate
		γ	
Stock 899*a* culture plated	1-0	0	$>10^7$
	1-3	3	45
Plate No. 1-0 printed	2-0	0	$>10^6$
	2-3	3	73
Spot picked on plate 2-0 corresponding to 1 colony on plate 2-3, stirred up in 3 ml. YEP, and plated	3-0	0	$>10^4$
	3-3	3	70
Printing, etc. repeated 7 times			
1 colony picked from 10-0. Grown up in YEP. Diluted 1×10^{-5} and plated	11-0	0	1.1×10^2
	11-3	3	1.1×10^2

It may be noted that spots corresponding to the location of colonies on the terramycin-agar were selected from the corresponding replica on YEP agar instead of from the master plate from which the replicas were made. All attempts to isolate the mutant from the master plate failed. This may be due to the fact that the master plate is necessarily 1 day older than the replica before the spot can be selected or because the sensitive colonies grow slightly larger than the resistant ones, and so overcrowd them on an old plate.

TABLE III

Comparison of a Sensitive Strain, a Resistant Strain Isolated by Replica Method, and a Resistant Strain from a Colony on 3 γ Terramycin/Ml. Agar

Terramycin/ml.	Lag, *hrs.*			Growth rate/*hr.*			Colonies/10^8 cells		
	Sensitive	Resistant		Sensitive	Resistant		Sensitive	Resistant	
		Replica	Overgrowth		Replica	Overgrowth		Replica	Overgrowth
γ									
0	0	0	0	1.9	1.7	1.7	$>10^7$	$>10^7$	$>10^7$
0.2	0	0	0	1.4	1.7	1.7	$>10^7$	$>10^7$	$>10^7$
0.5	0	0	0	1.0	1.7	1.7	50	$>10^7$	$>10^7$
1.0	10	0	0	0	1.6	1.6	30	$>10^7$	$>10^7$
2	10–20	0	0	0	1.5	1.6	14		
5	>24	0	0	0	1.1	1.1	18		
10				0	0.2	0.2	0	$>10^7$	$>10^7$
20				0				$>10^7$	$>10^7$
50				0				2	5

sufficient terramycin is added to decrease the growth rate, the fraction of mutant cells increases. The mechanism of this result is discussed in a following paper.

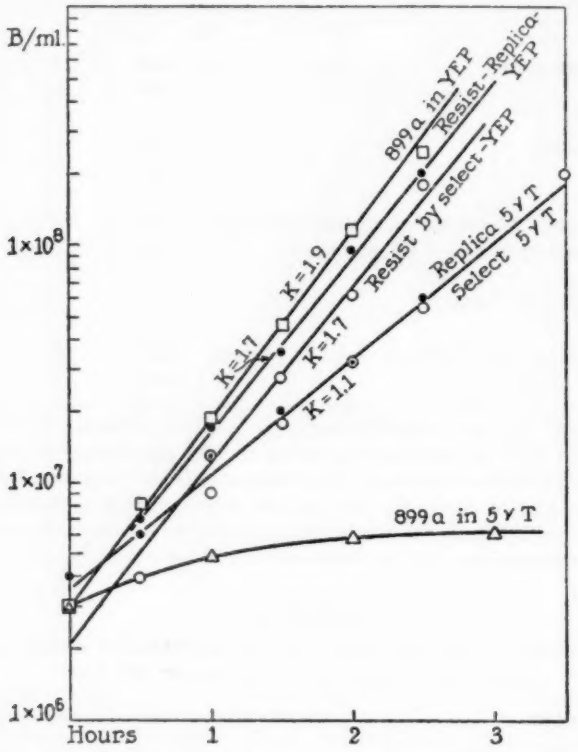

FIG. 2. Rate of growth of sensitive strain, resistant strain isolated by replica technique, and resistant strain isolated from overgrowth, in the presence of 0 and 5 γ terramycin/ml.

Origin of the Resistant Cells.—

The preceding experiments show that the resistant strain is the result of the overgrowth of the sensitive cells by resistant cells, but do not determine whether the resistant cells arise as mutants of the sensitive cells or are simply present as a mixed culture. The results of the experiments shown in Table IV, however, prove that the resistant cells arise from the sensitive cells and are not part of a mixed culture.[3] 1 ml. of the original suspension gave rise to more that 1 X 10^4 colonies on YEP agar, but no colonies on YEP agar containing 3 γ terramycin/ml. Ten colonies which grew from cells in the original suspension,

[3] This experiment is, of course, a modified form of Newcombe's (1949).

picked at random from a YEP plate and grown up in YEP, all contained resistant cells. These resistant cells must have arisen from sensitive cells. It is true that the total cell concentration is much higher in the final plating than in the

TABLE IV

Tests for Origin of Resistant Cells

899*a* in log growth in YEP diluted to about 1×10^8 cells/ml. 2 ml. in test tubes and shaken at 35° for 2 hours.

	Plate No.	YEP colonies/ml.	YEP + 3γ terramycin/ml. colonies/ml.
1 ml. plated on YEP ± 3 γ terramycin/ml.	1	$>10^4$	0
Diluted to about 100 B/ml. 1 ml. plated	2	62	0
10 colonies picked from YEP plate 2			
	Colony No.		
Suspended in 5 ml. YEP, grown up to about 5×10^7 B/ml., and 1 ml. plated on agar ± 3 γ terramycin/ml.	1	$>10^6$	3
	2	$>10^6$	15
	3	$>10^6$	8
	4	$>10^6$	4
	5	$>10^6$	100
	6	$>10^6$	5
	7	$>10^6$	12
	8	$>10^6$	25
	9	$>10^6$	2
	10	$>10^6$	40

TABLE V

Effect of Sensitive Cells on Colony Count of Resistant Cells

Sensitive cells—899*a* strain in log growth.
Resistant cells—Selected by replica—log growth in YEP.
Mixed as noted and plated on YEP and (YEP + 3 γ terramycin/ml.).

Sensitive cells/ml.	0	10^7	10^7	10^8	10^8
Resistant cells/ml.	100	—	100	—	100
Colonies/ml. on YEP	38	$>10^6$	$>10^6$	$>10^7$	$>10^7$
Colonies/ml. on 3 γ terramycin	42	0	46	20	70

original, but the total cell concentration does not affect the colony count of the resistant cells (Table V).

Determination of Resistant Cells by Colony Count and by Poisson Distribution.—If the resistant colonies are descendants of resistant cells present in the suspension from which the sample is taken, then the number of resistant cells in the sample as determined by colony count should be the same as that determined by means of Poisson's ratio for replicate cultures.

The results of a series of experiments designed to test this prediction are shown in Table VI. The number of resistant cells/sample by plate count is the same as that determined by Poisson's ratio. The experiments also show that the result depends only on the number of cells in the sample, and not on the concentration.

This result also makes it very unlikely that resistant cells can arise from sensitive cells without growth of the latter. If this process could occur, the number of colonies on the terramycin plate should increase with time, as the sensitive cells changed to resistant. This does not occur. The cells are not dead, because after 3 or 4 days many colonies do appear (owing to inactivation of the antibiotic) (*cf.* Bryson and Demerec, 1950), but these colonies are not resistant. The results of the Poisson ratio experiment confirm this. If the resistant cells

TABLE VI

Comparison of TR Colonies/Milliliter Suspension by Colony Count and by Poisson's Ratio

899*a* in log growth in YEP, about 10^8 B/ml. 1 ml. plated on 4 × 3 γ terramycin plates. Diluted to B/ml. noted in YEP + 2 γ terramycin/ml. and distributed in 10 to 20 tubes, shaken at 25° for 2 days, and number of tubes showing growth noted. Number of TR cells present in original sample calculated from Poisson's ratio.

Experiment	Sample	B/ml.	Total B	TR cells present/sample	
				From plate count of original suspension on 3γ terramycin-agar	From Poisson's ratio of total/negative tubes
	ml.				
3/20	3	1×10^6	3×10^6	2.0 ± 0.4	2.0
3/29	0.2	1×10^7	2×10^6	1.0 ± 0.2	1.2
3/26	5	1×10^5	5×10^5	0.2 ± 0.04	0.23
4/10	5	1×10^4	5×10^4	0.01	<0.2

are derived from sensitive cells without growth, then the number of tubes showing growth should increase with time. This does not happen either. Those tubes which do not show growth in 2 days (at 25°), never give resistant cell growth. After 3 or 4 days, growth does occur (owing to inactivation of the antibiotic) but the cells are sensitive. Englesberg and Stanier (1949) and Stocker (1949) also were unable to find any evidence of mutation in the absence of cell division.

The Ratio of Resistant/Sensitive Cells under Different Conditions.—Since the resistant cells are descended from sensitive cells, the ratio of resistant/sensitive must be 0 originally and increases with the growth of the clone. It soon reaches a constant value, however (owing to the difference in growth rates) (*cf.* Deskowitz and Shapiro, 1935; Novick and Szilard, 1950; and Northrop and Kunitz, 1956), and this equilibrium value remains remarkably constant either in repeated transfers or in the steady state apparatus as long as the total B concentration is fairly high (1×10^6/ml. or more).

Fluctuations in Ratio of Resistant/Total Cells in Separate Cultures.—If a series of tubes is inoculated with 1 or 2 cells/tube and allowed to grow, the number of cells/tube and also the number of either sensitive or resistant colonies/tube fluctuate more than the error of the analytical method (Table VII). Clones derived from a single sensitive colony also differ widely in the resistant/sensitive ratio at first, but agree fairly well after several transfers (Table VIII).

This variation may be due to slight differences in the conditions in individual tubes, or to differences in the lag, growth rate, or mutation rate of the individual

TABLE VII

Fluctuations in Cells/Milliliter and TR Colonies/Milliliter from Separate Tubes and from 1 Tube

899*a* in log growth. Diluted to 0.2 B/ml. in YEP. 10 ml. in test tubes. Shaken at 25°. B/ml. by turbidity. Colonies/milliliter and TR colonies/milliliter as noted. 1 sample from each tube. Remainder mixed together and 20 samples from mixture.

Time—25°		0	4 hrs	24 hrs.
B/ml.	From 20 separate tubes.....	[0.2] calc.		$180 \pm 30 \times 10^6$
	20 measurements on mixture....			$192 \pm 1 \times 10^6$
Colonies/ml.	Average of 20 plates from separate tubes...	0	5 ± 1	
	20 platings from mixture.	0	5.3 ± 0.3	
TR colonies/ml.	Average of 20 plates from separate tubes..	0	<1	32 ± 6
	20 platings from mixture.	0	<1	28 ± 1
TR colonies/10^8B	20 separate tubes.....			14 ± 1.6
	20 platings from mixture.			14 ± 0.5

cells from which the clones grow (Dean and Hinshelwood, 1952; and Northrop, 1955).

A large part of the fluctuation is due to the difference in growth rate of the clones, since the average TR colonies/10^8 B calculated from 20 separate tubes have a smaller deviation than either colonies/milliliter or TR colonies/milliliter from separate tubes.

Proportion of Terramycin-Resistant (TR) Mutants in Various Strains of B. megatherium.—The ratio of the TR mutants to total cells for various strains of *B. megatherium* is shown in Table IX. The strain isolated from a culture of *megatherium* which had become adapted to growth in ASCM contained a much larger ratio of resistant to sensitive cells.

The ratio for 899*a* is remarkably constant as long as the culture is grown in the same medium under the same conditions, as in the steady state apparatus (Northrop, 1954).

A culture of 899*a* growing in YEP in the steady state apparatus at a cell concentration of 5×10^7 gave resistant colony counts over a 6 weeks period

TABLE VIII

Variation in Ratio TR/Sensitive Cells from Different Resistant Colonies

899*a* streaked on 3 γ terramycin agar. 10 colonies transferred to YEP, grown up to 50×10^6 B/ml., and plated for TR colonies. Diluted 0.05/5. Let stand at 25°—18 hours. Diluted 1/10, grown up at 35° to 50×10^6 B/ml., and plated for TR colonies. Repeated.

Transfer No.	Colony No.	
	2	3
	Tr colonies/10^8 cells	
0	25	450
1	12	105
2	25	18
3	8	14
4	10	12

These were the two extreme results. The other 8 clones gave intermediate values at first. All the clones eventually gave 10 to 20 TR colonies/10^8 B. One culture in the steady state apparatus gave 300 to 500 TR colonies/10^8 cells for 12 days. The ratio then gradually decreased to the usual figure.

TABLE IX

Ratio TR Colonies/Total Cells.
Various Strains of B. megatherium in Log Growth in YEP

Strain	Terramycin/ml.			
	2.5γ	5γ	10γ	20γ
	TR colonies/10^8 B			
899*a*	25	25	20	0
AC 8*	1000	800	600	300
AC 82*	2000	1600	1500	200
SP*	1000–10,000	1000–10,000	250	100
KM	40		6	0
	(many very small colonies)			

* Strain isolated after adaptation of 899*a* to ASCM (Northrop and Murphy, 1956). These strains have lost their ability to grow rapidly on ASCM and have also lost the property of producing C phages.

which fell within the range of 10 to 20/ml., with the exception of two plates, one of 75 and one of 100 colonies.

The other strains, especially SP, show more variation in the resistant colony count in the range of 3 γ terramycin/ml. and the ratio is higher in very dilute suspensions.

Effect of Culture Media.—The ratio of resistant/total cells in cultures of *megatherium* 899*a* depends on the culture media (*cf.* Novick and Szilard, 1951). Fewer resistant mutants are present when the culture is grown in either ammonium sulfate or asparagine media. The phage concentration and gelatinase concentration are also much lower in these media than in YEP (Northrop, 1951). This effect is probably due to an increased difference in growth rates of

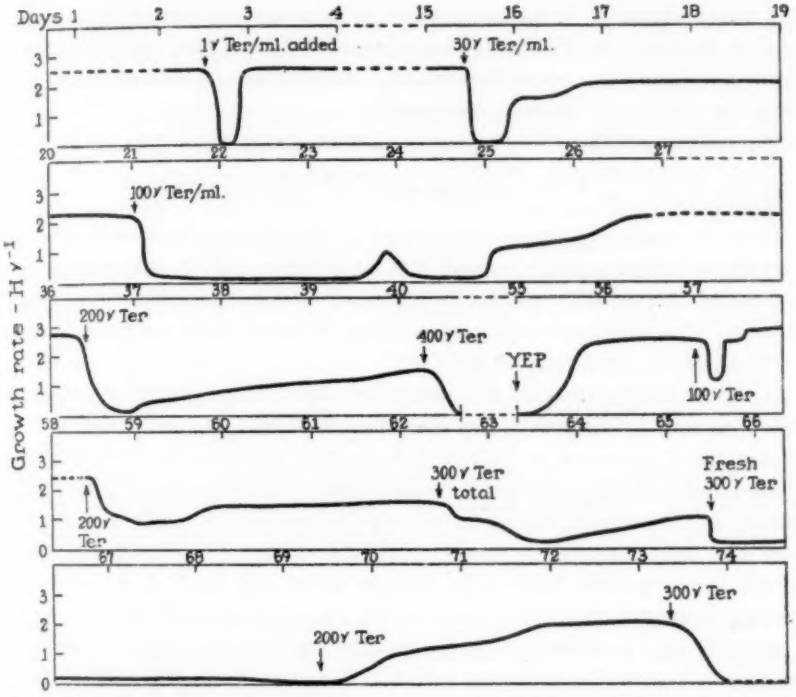

FIG. 3. Growth rate of *B. megatherium* cultures in YEP containing varying quantities of terramycin in steady state apparatus. The growth rate was calculated at 1 hour intervals from the kymograph record of the flow of culture medium as described previously (Northrop, 1954).

the sensitive and resistant cells (*cf.* Northrop and Kunitz, 1956). It is not due to a reversal of the mutation, since resistant cells may be grown in ammonium sulfate culture media for many generations.

Adaptation to Higher Concentrations of Terramycin

The result of an experiment in which 899*a* was grown in the steady state apparatus (Northrop, 1954) in the presence of varying concentrations of terramycin is shown in Fig. 3. After the addition of 1 γ terramycin/ml. to

the sensitive strain, the growth rate drops to 0, remains there for 4 hours and, then increases rapidly to its original value. The entire cycle requires about 9 hours. The growth tube contains about 200 resistant cells and 1×10^9 sensitive cells at the beginning of the experiment. Assuming that the sensitive cells stop growing as soon as the terramycin is added (this is not quite true) and that the resistant cells continue to grow at a rate of 2.5, it would require 7.7 hours for the resistant cells to constitute 10/11 of the cell population (Northrop, 1954). Actually, it required about 9 hours for the culture to resume its original growth rate. The calculated time should be slightly shorter since the sensitive cells continue to grow for a few hours after the terramycin is added, while the calculation assumes no growth.

The calculated time for the growth rate to change from 0 to 2.5 is 3 hours $\left(M_0/W_0 = \frac{1}{10}\ \frac{M}{W} = 10\right)$ and the observed time about 2.5.

When 30 γ terramycin/ml. were added to this culture, the growth rate again dropped to 0 very rapidly, stayed at 0 for 8 hours, and then increased to 1.5 in 4 hours. After that it gradually increased to 2.6. The entire cycle required 12 hours. The calculated time in this case is 11 hours, since the growth rate (at end of cycle) is 1.5 instead of 2.5.

The growth rate remained at about 2 for several days. 100 γ terramycin/ml. were then added. The growth rate decreased to 0 and remained there for 3 days. At this time it increased to 1.0 and then dropped rapidly to 0. This preliminary increase is due to the inactivation of the terramycin in the growth tube at 35° C. As soon as the terramycin had become partly inactive, the cells commenced to grow and fresh terramycin ran in from the storage bottle and stopped the growth in the growth tube. (The storage bottle was kept at 20° and so the terramycin remained active.) After about 24 hours, growth was resumed. It is probable that a resistant mutant appeared during the short growth period, and then overgrew the culture as before. 200 γ terramycin/ml. stopped growth for a few hours, but the growth rate returned slowly to 1.5 to 2.0. 400 γ terramycin/ml. stopped growth completely for nearly 2 weeks. At the end of this time. YEP was run in and the culture returned to its original growth rate of 2.5 in a few hours. After 30 hours in YEP, 100 γ terramycin/ml. were added as before. This time growth was resumed after 4 hours. Resistance to very high concentrations of terramycin decreases rapidly, at least as measured by the growth rate. Attempts to adapt the culture to 300 γ terramycin/ml. were repeated at intervals, but none was successful (*cf.* Wright *et al.*, 1953).

The successive steps in the adaptation are probably the result of the selection of successively resistant mutants, as in the case of penicillin resistance.

This is indicated by the fact that the lag time (*i.e.*, the time required for growth to commence again) is always about the same as that calculated on the assumption that the growth tube contains a few cells capable of growing

in the new concentration of antibiotic. In the cases in which growth did not commence within this time, or near it, it never increased. If no resistant cells were present in the growth tube at the time of the addition of the antibiotic, none appeared, therefore.

Loss of Resistance to Terramycin.—

Strains which have developed resistance to bactericidal substances lose their resistance sooner or later, when grown on favorable media. As a rule, the longer the culture has been grown in the presence of the antibiotic, the longer it retains its resistance. This is true, in general, in the present case, although the results are somewhat irregular owing to the small number of cultures. Strains adapted to 1 γ or to 100 γ terramycin/ml. were transferred daily in YEP, ASCM, filtrate from resistant or sensitive strains, and autoclaved filtrates. The strain adapted to 100 γ terramycin/ml. showed no loss of adaptation after

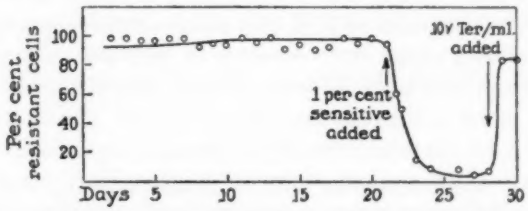

FIG. 4. Overgrowth of resistant by sensitive cells. Culture adapted to 150 γ terramycin/ml. in steady state apparatus and then grown continuously in YEP for 21 days. 1×10^8 cells/ml. 1×10^6/ml. sensitive cells added and culture plated for TR colonies as noted. 10 γ terramycin/ml. added.

125 transfers. The strains adapted to 1 γ terramycin became sensitive in YEP after 60 to 80 transfers, and in ASCM and the various filtrates somewhat more rapidly (*cf.* Foster and Pittillo, 1953).

This loss in resistance has been ascribed to back mutation, although it is extremely unlikely that such a mutation would give rise to a sensitive strain, identical with the original. The proportion of mutants in the reverted strains indicates that this is not the case, since the ratio of resistant/sensitive is much higher in some cases than in the original strain. The appearance of a few sensitive cells in a large population of resistant cells cannot be detected and direct proof of this mechanism is not possible at present. It can be shown, however, that if such a sensitive cell did arise in the mutant culture, it would overgrow the resistant cells and a sensitive culture would result (*cf.* Welsch, 1952).

The result of an experiment in which about 1 per cent of sensitive cells was added to a strain previously adapted to 150 γ terramycin/ml. and growing in YEP in the steady state apparatus is shown in Fig. 4. This resistant strain had grown for 2 months ($\simeq$ about 1500 generations) in the steady state apparatus,

in YEP, with no loss in resistance as determined by colony count on agar. After the addition of the sensitive cells, the resistant cells decreased in a few days to about 10 per cent, where they remained constant. If any sensitive cells appeared in the culture, therefore, they would probably have overgrown the mutant ones. In the 2 months period, about 3×10^{12} cells had grown in the cell, without the appearance of a sensitive one; the "back mutation" rate must evidently be very low.

Attempts to Transfer the Terramycin Resistance by Phage

The recent work of Freeman, 1951; Lederberg and Lederberg, 1953; Lederberg and Edwards, 1953; Fredericq, 1953; Stocker, Zinder, and Lederberg, 1953; Woolman, 1953, and others has shown that phage particles are a special kind of transforming principle (Gratia, 1936) and can transfer genetic properties from one cell to another. Hotchkiss (1953–54) found that resistance to antibiotics is transmitted by a specific transforming principle. In order to test this possibility, lysogenic cultures of KM (the phage-sensitive *megatherium* strain) were made by adding phage from sensitive or resistant cultures to the KM. The resulting lysogenic strains, however, were indistinguishable from the original KM strain, as far as the resistance was concerned. The fraction of the total phage responsible for the production of the lysogenic strain is exceedingly small, probably not over $1/10^6$, so that, unless all the phage particles were capable of transmitting the resistance factor, a positive result would not be effected (*cf.* Bryson and Demerec, 1955).

Experimental Procedure

Culture Media.—

Yeast Extract Peptone (YEP).—50g. Difco bacto-peptone + 5 gm. Difco yeast extract + 10 ml. M/2 NaOH, dissolved in 1 liter water. Boiled 20 minutes. 24 hours at 5°. Filtered; autoclaved.

Ammonium Sulfate Culture Medium (ASCM).—Northrop and Murphy, 1956.

Terramycin.—Crystalline terramycin HCl was kindly supplied by Charles Pfizer and Company. The terramycin was dissolved in 0.01 M HCl and filtered through a Seitz filter. The activity of the substance decreases slowly on storage at 5° and there is also some loss on filtration. The inhibiting effect of solutions prepared at different times, therefore, is not exactly comparable.

Terramycin Agar.—YEP agar melted, cooled rapidly to 50°, the desired concentration of terramycin added, and 15 ml. poured into petri dishes.

Colony Counts.—

Gratia's double layer technique was used. One-tenth volume of hot 2 per cent agar[4] was added to the suspension and then 1 ml. spread on the surface of the agar (Welsch *et al.*, 1953; Northrop, 1953).

[4] The concentration of agar in the superimposed layer should be just sufficient to prevent the layer from running or slipping when the plate is turned over. 0.2 per cent of the agar used in these experiments was sufficient to do this.

Control experiments showed that addition of the hot agar did not change the colony count. In case terramycin-agar plates were used, the colony count was the same whether or not terramycin was added to the suspension.

All colony plates were incubated at 35° for 18 hours (or at 25° for 48 hours). Longer incubation of plates containing terramycin results in the appearance of many small colonies. These colonies appear because the terramycin has been partially inactivated. They are not terramycin-resistant (Bryson and Demerec, 1950).

Cell Concentration by Turbidity.—

The turbidity was determined in a Klett-Summerson colorimeter adapted to 20 mm. tubes. The cell concentration was interpolated from the colorimeter reading by means of a standard curve prepared by plotting the cell count of a series of suspensions against the colorimeter reading. The cell concentration is nearly proportional to the colorimeter reading from 1 to 100 and then gradually falls off. During log growth in YEP the colony count is equal to the chains/milliliter and to 3 times the cells/milliliter (*cf.* Northrop, 1951).

Growth in Liquid Media.—

10 ml. of the culture was placed in 20 mm. tubes and the tubes shaken 200 to 300 times/minute in a water bath.

Steady State Apparatus.—

The steady state apparatus previously described (Northrop, 1954) was used, except for changes in the cell and wipers. The original cell foamed with some cultures (requiring a somewhat uncertain correction) (Northrop and Murphy, 1956) and mucoid organisms collected on the shoulders of the cell and on the rubber wipers themselves. The organism occasionally grew in the intake tube and in the culture medium reservoir bottle.

The cell and wipers shown in Fig. 5 obviate these difficulties.

10 per cent lactic acid and 1/5000 germ-i-tol are dropped slowly in the cell overflow through a hypodermic needle (*cf.* Northrop and Murphy, 1956) (except when a sample is taken). This prevents the accumulation of organisms outside the cell itself.

The air does not bubble through the liquid, and therefore, does not cause foaming.

The wipers were cut from a lusteroid test tube. They were cemented to the steel wire with cement made by dissolving lusteroid in acetone. The growth tube is filled with glycerine before autoclaving. This prevents the lusteroid wipers from becoming opaque and brittle. These thin wipers cause much less foaming than do the segments of rubber tubing used originally, and retain fewer organisms. The wipers are run as rapidly as possible (usually 300 to 400 strokes/minute) without causing foam; at half-hour intervals a microswitch on the kymograph axle causes the wipers to operate 600 to 700/minutes for 15 to 20 seconds. This keeps the cell walls perfectly clean even with very mucoid organisms, but some cells still adhere to the wipers in spite of the violent stirring. The number of cells which come from the wipers may be determined by running the culture media continuously through the cell at a rate of about 100 ml. per hour until the cell concentration in the overflow remains constant. This corresponds to a wash out rate of 5/hour (since the cell volume is about 20 ml.). This is 2 (or more) times the growth rate of the bacteria and so the concentration of cells in suspension

in the tube would decrease at a rate of (5 − growth rate) and would rapidly approach 0. If cells are constantly growing and being liberated from the cell walls or wipers, however, the cell concentration in the overflow will not approach 0, but some constant value and this value represents the cells coming from the walls or wipers. With the rubber wipers, these cells amounted to about 1×10^9 per hour. With the lusteroid

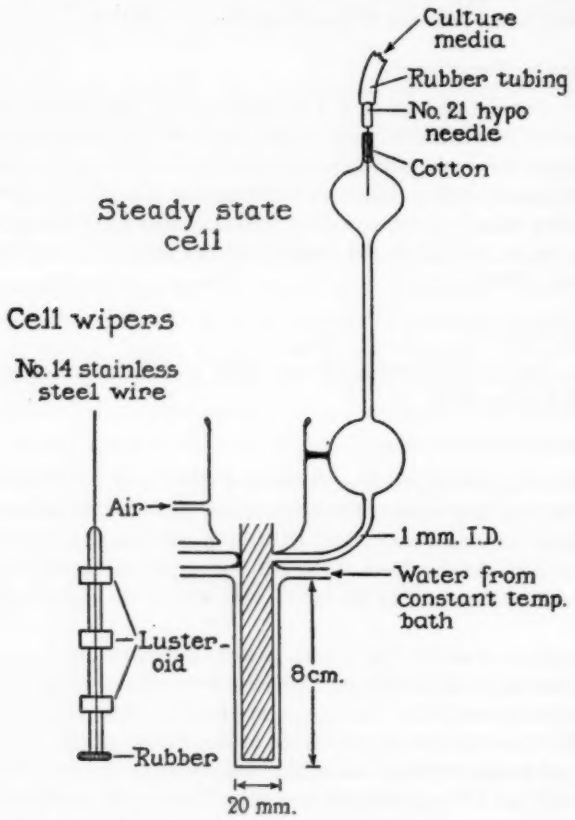

FIG. 5. Cell for use with steady state apparatus. Wipers for cell cut from lusteroid test tube. The end wiper is a segment of rubber tube.

wipers about 1×10^8 cells/hour were delivered with mucoid organisms. A culture growing in the cell at a rate of 2/hour and a concentration of 1×10^8/ml. (usual conditions) delivers $20 \times 10^8 \times 2 = 4 \times 10^9$ cells/hour. The cells derived from organisms growing on the wipers are therefore 2 to 3 per cent of the total. This is negligible if the cells do not differ *qualitatively* from those in suspension, but if they are different qualitatively, serious errors may result. In the present experiments the ratio of TR/total cells is the same in cultures derived from the wipers, as from the suspension in the cell.

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INTRACELLULAR RECORDING FROM THE GIANT SYNAPSE OF THE SQUID*

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(Received for publication, September 10, 1956)

Several authors have described intracellular recording from the post-junctional cell in synaptic regions, for example, in the neuromuscular junction (Fatt and Katz, 1951, 1953), in the brain and spinal cord (Brock, Coombs, and Eccles, 1952; Woodbury and Patton, 1952; Tasaki, Polley, and Orrego, 1954; Tasaki, Hagiwara, and Watanabe, 1954; Araki and Otani, 1955; Coombs, Eccles, and Fatt, 1955; Frank and Fuortes, 1955, 1956), in the sympathetic ganglion cell (R. Eccles, 1955), the electroplaque of electric fish (Albe-Fessard and Buser, 1952; Albe-Fessard and Chagas, 1954; Altamirano, Coates, and Grundfest, 1955), and in the large monopolar ganglion cells of certain invertebrates (Tauc, 1955; Arvanitaki and Chalazonitis, 1956; Hagiwara and Watanabe, 1956). Only in the nerve-muscle junction of the frog and the electroplaque of the eel, however, have the records been obtained from a region of the postjunctional cell known to be near the prefiber ending and only in the former is the junction supplied with but one input fiber. The giant synapse in the stellate ganglion of the squid between the giant fibers of the second and the third order offers a particularly favorable object for such recordings. Especially valuable is the opportunity to place microelectrodes internally in the presynaptic fiber close to the ending as well as in the postsynaptic unit, close to the junction. This preparation was studied some years ago with macroelectrodes on the surface of the ganglion (Bullock, 1948). A preliminary account of the present observations has appeared (Bullock and Hagiwara, 1955).

Materials and Methods

The stellate ganglion was isolated together with several centimeters of preganglionic nerve and postganglionic (last stellar) nerve from the squid *Loligo pealii*. The dissection was carried out in running and aerated, chilled sea water at about 14°C. After testing for the presence of normal transmission, the preparation was mounted on a glass slide inclined at a slight angle to facilitate insertion of the micropipette nearly parallel to the long axis of the nerve. The ganglion rested in sea water while the ante-

* Aided by grants from the National Institute for Neurological Diseases and Blindness (B-21) and the University of California.

rior end of the prenerve and the posterior end of the postnerve were lifted up into air for stimulation and external recording respectively. The whole vessel was surrounded by ice water, keeping the temperature between 15 and 20°C.

When the preparation is properly oriented and in a favorable light it is possible to see not only the tributary processes of the third order giant fiber but also the terminal digitations of the presynaptic second order giant (Fig. 1). The glass micropipettes filled with 3 M KCl were inserted, after a minimum of dissection, into the pre- or postsynaptic fiber approximately as shown in Fig. 1 and pushed longitudinally from 1 to several millimeters towards the region of the synapse. Long shank electrodes of 10 to 30 megohms resistance were used leading to cathode follower input units of 10 micromicrofarad capacity or less. Following the input stage, amplification was provided to a final sensitivity of 1 mv. per inch.

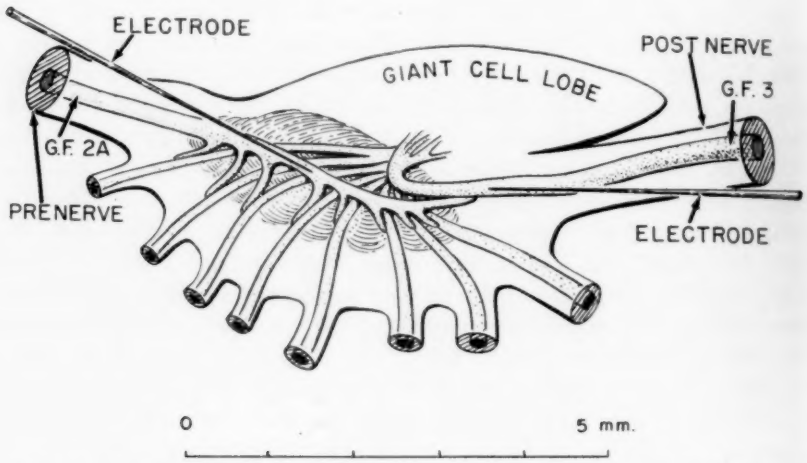

FIG. 1. Slightly diagrammatic representation of the preparation used, drawn to scale.

The preparations lasted up to several hours and those which failed early seemed to be associated with excessive effort to clean the preganglionic nerve of small fibers in order to prevent small fiber spikes from entering the ganglion. This precaution proved to be unessential since intracellular records showed little or no sign of deflection attributable to small fibers.

RESULTS

Form of the Postsynaptic Response to a Presynaptic Impulse.—The fresh synapse transmits in a one-to-one manner, and we therefore record a full spike. This looks very much like the ordinary spikes in the axon, but appears to be somewhat longer in duration. Measured from the first inflection to the beginning of the undershoot, values of 1.5 to 2 msec. are common at 18–20°C. We have always seen an undershoot in this position of recording, but it varies greatly in amplitude from about 2 to 5 or more millivolts.

The synaptic potential has been studied by fatiguing the spike until it drops out in an all-or-none manner. The potential that remains, which we have regarded as the synaptic potential, is not higher than 10 mv. and may be as small as 1 mv. as recorded at this distance. Since the process of fatigue has decreased the rate of rise and the amplitude of the synaptic potential, the fully normal unfatigued synaptic potential is not available to us for observation (*cf.* Kao and Grundfest, 1955 on squid axon local response).

The form of the synaptic potential recorded 2 mm. from the junction, that is much less than 1 space constant away is seen in Fig. 2. Its rise requires about 1 to 1.5 msec. and its fall about 2.5 to 3 msec. at a temperature near 21°C. The falling phase does not fit a simple exponential form. There is often a small undershoot which also would not be expected of a purely passive potential in

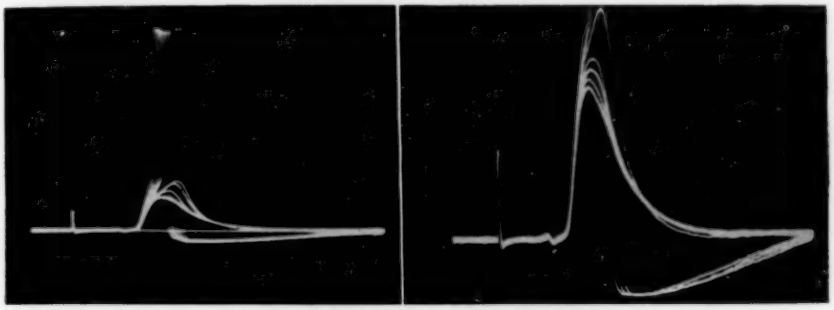

FIG. 2. The orthodromically transmitted response at the moment of greatest alteration with fatigue under high frequency stimulation, recorded inside the postfiber, at lower (left) and higher (right) amplification. Calibration, 10 mv. and 5 ms.

a simple leaky condenser cable. Further stimulation, bringing on further fatigue, seems to involve not only a decrease in the rate of rise of the synaptic potential, but a shortening of the duration of the rising phase, and a slowing of the falling phase. Every one of the features mentioned is quite variable from preparation to preparation. Thus no noticeable undershoot is recorded in some cases. There may be a considerable decline in amplitude with fatigue without any noticeable change in the duration of the falling phase. The crest time may not shift considerably to the left in some cases.

A few experiments were carried out in which an intracellular electrode in the prefiber close to the synapse was used as stimulating electrode. An indication that the amplitude of the prefiber action can determine the postfiber response was seen. It was found that we had control of the postsynaptic response amplitude. Over a small range of stimulus intensity transmission occurred but without setting up a postspike, only a postsynaptic potential of variable size. A slightly stronger shock elicited a postspike.

With the exceptions of the special experiments described in the last para-

graph, all effects of preganglionic nerve stimulation upon the third order giant fiber were all-or-none. Young (1939) described two giant fibers in the preganglionic nerve which end in relation to the third order giant fiber in the stellate ganglion at different levels, one closer and one farther from the cell bodies. As in the earlier experiments (Bullock, 1948) no physiological sign could be found of two separate effects. We have no direct evidence of which junction we are

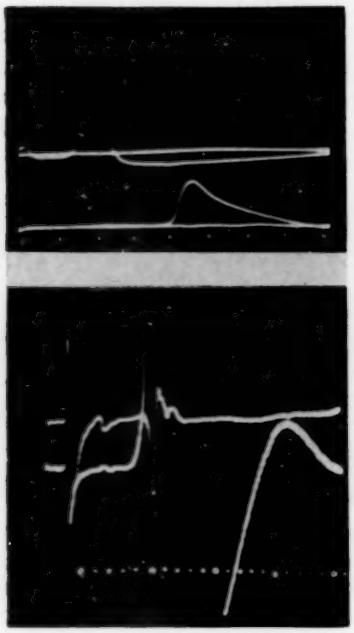

FIG. 3. Pre- and postsynaptic potentials recorded simultaneously. Upper, both recorded intracellularly at the positions shown in Fig. 1, the prespike at low amplification is a full spike, the postresponse, at higher amplification is a synaptic potential after the spike has been eliminated by fatigue. Lower, the postresponse, here a full spike going off scale, is recorded intracellularly on the lower beam; the upper beam is a steel microelectrode extracellularly recording both pre- and postspikes. Time marks, 1000 per sec.

studying but believe it more reasonable to attribute the observed synaptic potentials to the distal one, as shown in Fig. 1.

Synaptic Delay.—The suggestion has frequently arisen in the literature that in at least some preparations the true delay after correcting for prefiber conduction time would prove to be vanishingly small. Accordingly special effort was made in the present material to test this point. The evidence obtained is contrary to this suggestion. Simultaneous recording with microelectrodes inside the prefiber and inside the postfiber (Fig. 3) shows a delay between the

prespike and the postsynaptic potential of some 1 to 2 msec. The tips of these two electrodes are about 2 mm. apart and both are in fibers of large diameter. In the case of the postfiber recording, we can reasonably assume that at high gain the first deflection we see represents activity in the very region of initiation of postunit response, even if it is at some distance proximal to the electrode. The time of activity in the terminals of the presynaptic fiber is not so easy to establish, as distinct from preterminal activity. The internal electrode in the presynaptic fiber gives an approximation. We have measured from the crest of the prespike (Fig. 3) but there would be a considerable delay even if we measured from the beginning of the undershoot.

We have probed around the ganglion with an extracellular microelectrode of steel sharpened to 3 to 10 micra, hence visualizing on the same electrode both pre- and postspikes. In some cases the glass micropipette electrode has been inserted into the postunit while the steel electrode was in place. The delay as recorded is not sensitive to the position of the extracellular recording electrode. The latter may be in any part of the ganglion. At 15–20°C. the postsynaptic deflection starts 1 to 2 msec. after the prespike crosses the base line. The results agree with those obtained by intracellular recording. They also agree, within the range of variation, with the data reported in 1948 which were obtained by surface recording with macroelectrodes on preparations in air and hence assigned to the points of emergence of the pre- and postnerves from the ganglion. In the present measurements with extracellular steel microelectrodes there is no reason to assign the records to the same points because the preparations were immersed in sea water. Furthermore, in simultaneous records with intracellular electrodes in the postfiber and extracellular electrodes in the ganglion, as in Fig. 3, it can be seen that the discrepancy in time of first deflection of the postsynaptic response is only a small fraction of the whole delay—and can largely be attributed to the difference in amplification. If the same is true of the prespike, then there is a negligible correction for conduction in the extracellular microelectrode records. We did not succeed in simultaneously recording with this electrode and one inside the prefiber.

The exact value of synaptic delay is not the same from preparation to preparation but there is always a distinct delay.

Fatigue cannot be the main explanation of the variation. We find that fatigue produced by repetitive stimulation alters the rate of rise of the postsynaptic potential and hence the spike time but not the delay measured to the beginning of the postsynaptic deflection, in agreement with the 1948 report.

Amplitude of Prespike Seen across Postsynaptic Membrane.—A still better way to observe the delay would be to record the electrotonic sign of the prespike across the postsynaptic membrane. As long as it was possible to believe that there may be no real delay, it could not be expected with assurance that this prespike would be distinguishable from the postsynaptic potential. But now

we believe it must be separately discernible and the main question whose answer cannot be predicted is how large it will be. In most of our records, when the amplification permits recognition of signals as small as 1 mv., there is no sign of a prespike inside the postfiber (Fig. 4). In some of the high amplification records we see a deflection which must represent the prespike (Fig. 2, right, and 3, lower) but it is only 100 to 300 μv. which is similar to the amplitude

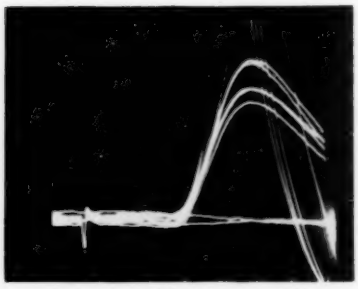

FIG. 4

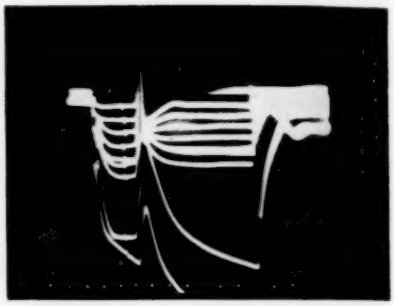

FIG. 5

FIG. 4. Intracellular record in the postunit at high amplification showing the usual finding of no detectable deflection attributable to the field potential of the arriving presynaptic spike. Calibration in millivolts and milliseconds.

FIG. 5. The effect of hyperpolarization of the postsynaptic membrane by applying a long square pulse through one penetrating electrode, recorded inside the postunit by a second electrode. The first deflection is the hyperpolarizing potential. Several sweeps are superimposed at different levels of membrane polarization from 3 mv. (above) to about 50 mv. (below, subtracting an artifactual component). The second deflection is the stimulus artifact of the shock to the prefiber. The third is the postsynaptic response—a spike in this unfatigued case, until high polarization is reached. At the off of the polarizing pulse another spike occurs as a consequence of the direct stimulus to the postmembrane. Calibration, 10 mv. and 2 ms.

of prespike recorded with an extracellular steel electrode. Smaller spikes could not be reliably discerned due to the noise level.

The reference electrode was not just outside the postunit membrane but far away, so that the true field voltage across the membrane is probably much smaller than that observed. After allowing for the decrement due to the 1 to 2 mm. between synapse and intracellular recording electrode, we still can estimate that the prespike is less than 1 mv. and often is not visible at all.

Polarization of the Synaptic Membranes.—At the junction of nerve and muscle Fatt and Katz (1951) in the frog and Hagiwara and Watanabe (1954) in the insect, found the endplate potential to be greatly enhanced by hyperpolariza-

tion of the postsynaptic membrane. In the motoneuron of the spinal cord Coombs, Eccles, and Fatt (1955) found no enhancement of e.p.s.p. amplitude.

Fig. 5 shows one record from a typical experiment on the squid synapse. We sometimes saw a small enhancement of the synaptic potential and in other preparations saw some depression, especially of the rate of rise, when hyperpolarizing up to 90 mv. above resting membrane potential. In these experiments the polarizing electrode lies close to the recording electrode, inside the postfiber and between 1 and 2 mm. from the synapse. Since the space constant is longer than this, a significant fraction of the imposed potential must be felt at the synapse. Unless we can assume that the fraction of the synaptic potential felt at the recording electrode has decremented more steeply under polarization than before, the near constancy of size of the observed potential indicates a real difference from the other junctions mentioned.

Hyperpolarization during transmission of the unfatigued spike first replaces the undershoot with a slowly increasing inside negativity, then at some critical level blocks the spike. Up to 80 mv. could be imposed, adding that much to the resting membrane potential, before blocking the spike, in some cases. The *absolute* level of membrane potential at which the synaptic potential flared up into a spike became higher than normal, even higher than the initial resting potential; *e.g.*, the height of the *potential change* from the 40 mv. hyperpolarized base line to flare up of spike was about 30 mv. in Fig. 6. This is probably somewhat above the unpolarized value although in the same experiment the preparation was too fresh to show a step before polarization. The spike exceeded 200 mv. in peak amplitude, in some cases.

Imposed potentials in the opposite polarity, decreasing the membrane potential, slightly decreased the synaptic potential if the base line had been shifted more than 10 mv. Reversal of the sign of the synaptic potential was not seen but greater depolarizations were not studied.

Interaction of Synaptic Potential and Antidromic Spike.—If an antidromic spike is allowed to enter and occupy the synaptic region just ahead of the beginning of a synaptic response to an orthodromic impulse arriving in the presynaptic fiber, the contribution of the latter depends on the phase of the former during which it occurs. We find no addition or subtraction of any appreciable potential to the peak or early falling phase of the antidromic spike. Only if the synaptic potential is elicited late enough that it can rise out of the later falling phase or the phase of undershoot is an addition to the potential seen (Fig. 6). It begins minimally and contributes more potential as it occurs later, but may not be fully normal in amplitude for more than a millisecond after the peak of the antidromic spike. The synaptic delay is considerably increased and progressively more so as the synaptic potential arises earlier in the recovery phase of the antidromic spike.

The question has been raised whether synaptic potentials can occur during

the absolute refractory period to direct electrical stimulation (Altamirano *et al.*, 1955; Grundfest, 1956). We cannot answer this by direct test in this preparation but only by analogy with the axon. We cannot say that a direct stimulus applied through our internal electrode or through external electrodes in the vicinity of the junction really tests the excitability of the synaptic membrane

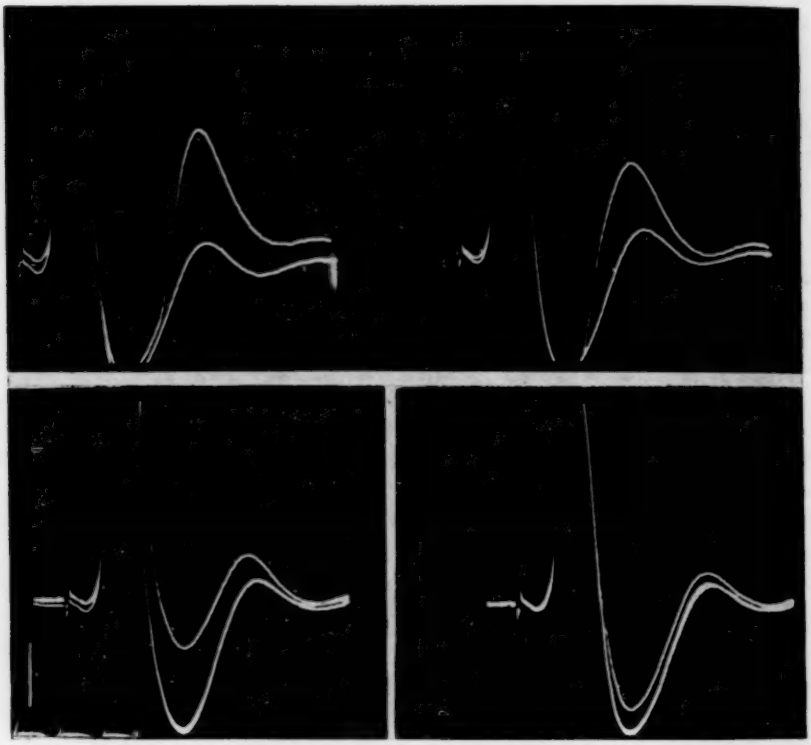

FIG. 6. Interaction of orthodromically transmitted synaptic potential and antidromic spike. The latter arrives first in each case and the former at an earlier phase in each successive picture. A large synaptic potential can rise out of the undershoot and a small one from the later part of the falling phase. Calibration, 1 mv. and 3 ms.

rather than an adjacent region of axonal membrane. By analogy with the axon the membrane would be refractory even to strong stimuli during the falling phase of a spike and we can therefore regard the synaptic potential as occurring during a time when that membrane is probably refractory to electrical stimuli.

Absence of "Synaptic Noise" and "Miniature Potentials."—In this preparation we have not seen any sign of "synaptic noise" or of "miniature potentials" at high amplification, in the absence of intentional prenerve stimulation.

DISCUSSION

One of the most interesting points discovered is the control of the postsynaptic response by the amplitude of the presynaptic action. By directly stimulating through the intracellular electrode in the prefiber close to the synapse, it was possible to vary the size of the postsynaptic potential over some range. At a critical intensity a postspike appeared. The stimuli used could not have excited the postfiber directly and must have elicited graded prefiber action. From the present evidence we cannot say whether the prefiber response was subthreshold or all-or-none. It is of the most general neurological interest to inquire whether specific neuronal interaction can occur without impulses; that is, whether a presynaptic neuron can excite at least a postsynaptic potential although itself not propagating a full all-or-none spike but only undergoing a graded, local response. It has been proposed (Bullock, 1953) that such interaction may be an important part of normal excitation by synaptic pathways, particularly in gray matter among short axon neurons and in invertebrate neuropiles in which afferent and efferent processes of a given neuron may be close together. If the present case proves to involve subthreshold prefiber activity, it would represent such a transmission without impulse, although, in all likelihood this is not a normal event for this particular junction.

With respect to synaptic delay, the evidence obtained forces us to the conclusion that there is a real delay between the arrival of the presynaptic impulse at the junction and the initiation of the postsynaptic potential, which cannot be attributed to conduction time in the presynaptic terminals. We can add nothing from these results concerning the suggestion (Bullock, 1952) that a considerable part of the synaptic delay is consumed inside the postsynaptic unit after transmission time has been completed and before visible response begins, but we can confirm the earlier report that there is no necessary increase in the synaptic delay with fatigue induced by repetitive stimulation.

Since the last conclusion removes the possibility that the presynaptic impulse would be lost in the beginning of postsynaptic response as seen inside the postsynaptic unit, it becomes possible to look for signs of the field potential of the presynaptic impulse across the postsynaptic membrane. The evidence reported indicates that this must be smaller than the noise level of our system in most cases, in a few however, reaching an amplitude of 100 to 300 microvolts. Since this is an order of magnitude or more smaller than the potential across the membrane necessary to stimulate the fiber with an abrupt electric shock, we must conclude that the available transmembrane current provided by the presynaptic impulse is too small for any known means of exciting the postsynaptic unit electrically. Terzuolo and Bullock (1956) have shown the physiological effectiveness of even such small potential gradients as these—of the order of tens of microvolts, in other preparations, but the essential condition for such

high sensitivity to potential fields seems to be that the cell is already active and this weak field modulates the frequency of firing. They report that the activation of a silent cell, even one which has been poised fairly close to firing, requires currents of at least 20 times greater intensity.

Del Castillo and Katz (1954) and Brock, Coombs, and Eccles (1952) also show small or indetectable presynaptic spikes in their records from intracellular electrodes in postunits, motor endplates in muscle, and ventral horn cells, respectively. It is impossible in these cases to establish the true delay, independent of conduction time in the terminals. The small prespike sometimes seen may be recorded at a discontinuity in the extracellular resistance some distance from the junction; *e.g.*, where the prenerve enters the main mass of tissue. The true prespike at the junction could therefore be obscured by the rising phase of the endplate or postsynaptic potential. These authors therefore, correctly, did not make a special point of the evidence against electrical transmission from the absence of a noticeable prespike across the postsynaptic membrane in those preparations.

Del Castillo and Katz (1954) placed weight instead upon the absence of detectable sign in the endplate of an electrotonic potential imposed on the nerve terminal adequate in amplitude to alter the frequency of miniature endplate potentials. While we now agree with their conclusion, it may be said that the argument suffers from the finding (Terzuolo and Bullock, 1956) that extremely small voltage gradients are adequate to modulate frequency of already active cells. Even the external field across the unit directly influenced (presumed to be the prefiber terminals) is probably less than 100 microvolts, the potential across any given membrane in the field still smaller. Thus the absence of visible potential change upon imposing the polarizing current is expected and does not represent a special attenuation across the postmembrane.

An attenuation of the normal prespike does occur, according to the present evidence, such that its field is weaker than 300 microvolts across the nearby postsynaptic membrane and this would appear to be a crucial argument against electrical transmission.

Hyperpolarization of the postsynaptic membrane has produced surprisingly large spikes (200 mv.) before blocking. There is some decrease in the overshoot, but it is not in proportion to the increase in membrane potential. The potential level, where the spike flares up out of the synaptic potential, shifts with hyperpolarization towards higher membrane potential levels. The postsynaptic potential is either slightly altered or not altered in amplitude—in agreement with the results of Coombs, Eccles, and Fatt (1955) but in contrast to the condition reported by Fatt and Katz (1951), and Hagiwara and Watanabe (1954). If this finding is correct it suggests that in the present preparation, the synaptic potential does not represent a short circuit across the membrane or have an equilibrium potential at which its amplitude becomes zero.

Also, in contrast to the neuromuscular junction and to the results with

the electroplaque of the electric eel, in the present synapse—a presynaptic impulse cannot cause any additional potential change on the crest or early in the falling phase of an antidromic spike in the postunit but begins to appear only late in the falling phase or during the undershoot. However, it does appear that the earliest part of this phase of possible addition of synaptic potential to falling phase of spike coincides with the period of complete refractoriness of postsynaptic axon. This rests upon analogy with the axon for we cannot directly test the excitability of the synaptic region to electric shocks at various phases of the recovery after an antidromic spike with any assurance that we are not exciting regions of the axon adjacent to the synapse. This finding is in agreement with the report of Fatt and Katz (1951) and of Grundfest (1956), although it is not as marked in the squid.

SUMMARY

1. Recording with glass micropipette electrodes inserted close to the synaptic region, in the presynaptic and in the postsynaptic fibers of the giant synapse in the stellate ganglion of the squid, has been accomplished.

2. The forms of the spike and of the synaptic potential are very much like those reported earlier (Bullock, 1948) from macroelectrodes. The crest time and the rate of fall are labile and depend on the state of fatigue, though the time of initiation of the postsynaptic potential does not.

3. It is concluded after examination of both intra- and extracellular recordings that there is a real synaptic delay of the order of 1 or 2 milliseconds at 15–20°C.

4. There is sometimes a very small and sometimes no visible deflection in the intracellular postsynaptic record attributable to the presynaptic spike. It is concluded that transmission cannot be electrical.

5. The amplitude of the postsynaptic potential can be controlled over some range by the amplitude of the presynaptic potential.

6. Hyperpolarization of the postsynaptic membrane results in increase in amplitude of spikes up to 200 millivolts, in increase in the membrane potential level at which the spike flares up, but in no considerable change in the amplitude in postsynaptic potential.

7. The postsynaptic potential can add to the late falling phase and the undershoot of an antidromic spike in the postfiber but cannot add to the crest or early part of the falling phase. The earliest part of the antidromic spike during which the postsynaptic potential can add is probably a period of refractoriness to electrical shock by analogy with the properties of the axon.

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THE COURSE OF RESPIRATION DURING THE LIFE CYCLE OF CHLORELLA CELLS*

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(Received for publication, July 13, 1956)

INTRODUCTION

The hereditary, environmental, and developmental sources of variability among organisms are of constant concern to students of cell physiology. Though heredity and environment are usually considered, far less attention has been devoted to eliminating the variability caused by the growth and development of the cells. In this paper the importance of cell age, or of the developmental stage of a cell as it influences its metabolic characteristics, is emphasized, as studied on synchronized algal suspensions.

Material and Methods

The work was done with the high-temperature strain, 7-11-05, of *Chlorella pyrenoidosa* (Sorokin and Myers, 1953 and 1954). To induce synchronization in cell development algal suspensions were grown at 39°C. under diurnally intermittent illumination: 9 hours' light and 15 hours' dark. After 9 hours of continuous illumination the wall of the mother cells (at the beginning in only a few cells) began to rupture. This rupturing, with the liberation of autospores, is referred to in later discussion as cell division. If after 9 hours of illumination a cell suspension is darkened, growth in mass is interrupted in all cells, but the divisions continue. In the absence of organic carbon the growth of liberated autospores is postponed until after illumination is started. After 3 to 4 cycles of this light: dark regimen an algal population contains at the end of a dark period up to 99 per cent of the cells as small autospores. When illuminated, cells of such an algal suspension start growing simultaneously. To maintain synchronization in cell development the described regimen of light:dark was kept throughout the entire period during which a certain algal culture was the source of cells for the studies reported in this paper. The developmental stages of the cells are denoted by the lapse of time in hours from the start of growth (start of illumination) to the moment when growth was interrupted by harvesting cells from the culture chamber.

Cells were grown in a continuous culture apparatus (Myers and Clark, 1944) under

* Aided by grants from the Carnegie Institution of Washington, and The Rockefeller Foundation.

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conditions permitting the maintenance of constant temperature, illumination, and nutrient supply throughout the investigation. Carbon dioxide was supplied by bubbling a 3.5 per cent CO_2—in air mixture through the algal suspension during both the light and dark periods; temperature was maintained at 39°C.; light intensity from tungsten bulbs as measured on the surface of the culture chamber was near 4,000 lux. Density of the algal suspension was kept at about 1 mm.^{3} packed volume of cells per 1 ml. of suspension. In grams per liter the culture medium contained: KNO_3—1.25, KH_2PO_4—1.25, $MgSO_4 \cdot 7H_2O$—1.00, $CaCl_2$—0.0835, H_2BO_3—0.1142, $FeSO_4 \cdot 7H_2O$—0.0498, $ZnSO_4 \cdot 7H_2O$—0.0882, $MnCl_2 \cdot 4H_2O$—0.0144, MoO_3—0.0071, $CuSO_4 \cdot 5H_2O$—0.0157, $Co(NO_3)_2 \cdot 6H_2O$—0.0049, ethylenediaminetetraacetic acid (as a chelating agent)—0.5. The pH of the medium was 6.8. Sterile conditions were maintained in the growth chamber.

Respiration studies were done by means of the direct Warburg method. An algal suspension was harvested from the continuous culture apparatus. An aliquot of this suspension was centrifuged and the deposit resuspended in a three salt suspending fluid containing in grams per liter: KNO_3—1.25, $MgSO_4 \cdot 7H_2O$—1.00, KH_2PO_4—2.50. In studies of glucose respiration 10 gm. of glucose were added to the above solution. After this preparation the cell suspension was stirred by bubbling air through it. A pair of vessels was used to determine both oxygen and carbon dioxide exchange. Into each vessel were measured 2.5 ml. of algal suspension containing about 20 mm.^{3} of algal cells. The central well of one vessel contained 0.25 ml. of 15 per cent KOH. Data from at least two pairs of vessels were averaged to obtain a single point for the figures. When only oxygen consumption was determined a single vessel method was used. KOH was placed in the central well and at least two vessels were used to obtain the average values presented in figures and tables. Vessels were shaken in the bath at a speed of 120 oscillations per minute. Temperature in the bath was 39°C.

The time factor was of considerable importance and was recorded as routine. It took about 50 minutes from the moment of harvesting cells until the respiration vessels were placed into the bath. During this time algal cells were at room temperature of about 26°C. The first readings were taken after an equilibration period of 10 minutes. Readings at zero time (the moment of placing vessels into the bath) were obtained by graphic extrapolation.

Endogenous respiration was studied at two levels of pH, 4.5 and 6.8. In measuring glucose respiration at pH 6.8 two techniques were employed. In one case glucose was added to the suspending fluid before cells were transferred into it. In this way the time was cut to a minimum during which cells were deprived of organic carbon after having been harvested from the growth chamber. In the second case cells after centrifugation were taken into a suspending fluid from which glucose was absent and allowed to undergo endogenous respiration. After readings on endogenous respiration had been taken for 60 minutes, glucose was added from the side arm, and readings on glucose respiration were started. Rates of endogenous respiration, and of glucose respiration of cells introduced into the glucose medium after centrifugation, are given for the intervals of time 0 to 30 minutes after placing flasks into the bath. When glucose was added after 60 minutes of endogenous respiration, rates of glucose respiration are for 0 to 30 minute intervals after the addition of glucose.

RESULTS AND DISCUSSION

In Table I are listed respiratory quotients (CO_2/O_2) for cells of seven successive developmental stages. In each case measurements are given for two intervals of time of the same respiration experiment: 0 to 30 and 50 to 80 minutes after placing manometric vessels into the bath.

In general, the respiratory quotient is rather stable in the course of cell development, an observation recorded also by Nihei *et al.* (1954). Some increase in the respiratory quotient after exposure of cells to light was due to the reduction of nitrates (Cramer and Myers, 1949). The relative stability of the respiratory quotient both in the course of cell development and during a respiration experiment allows us to base the following discussions largely upon the rates of oxygen evolution for which more extensive data are available.

TABLE I

Respiratory Quotients (CO_2/O_2) for Cells of Successive Developmental Stages of C. pyrenoidosa, Strain 7-11-05

Developmental stage of the cells Duration of illumination or darkness in growth chamber in hours		R.Q. for intervals of time during one respiratory experiment	
Dark	Light	0 to 30 min.	50 to 80 min.
(1)	(2)	(3)	(4)
15	0	1.3	1.2
15	1	1.5	1.2
15	3	1.5	1.2
15	4	1.3	1.2
15	6	1.5	1.4
15	9	1.5	1.5
5	0	1.4	1.3

As seen in Fig. 1 there is practically no difference in the rates of endogenous respiration measured at two different pH values. Rates for glucose respiration, as is usually the case, are considerably higher than those for endogenous respiration but in general they follow the same pattern in the course of cell development. Other conditions being equal, cells after being harvested from the growth chamber continue to respire at a rate depending on the supply of respiratory material available to the cell. In presenting data on endogenous respiration the time interval at which they have been recorded is of utmost importance. This change in the rate of endogenous respiration with the progress of the experiment, and the importance of the time factor in recording manometric readings acquire even more significance in work with cells of different developmental stages, subjected to illumination for different lengths of time.

In Fig. 2 are presented time course curves for the rates of endogenous respira-

tion of cells of six successive developmental stages. Centrifugation, transferring cells into suspending fluid, aerating the algal suspension, and filling the respiratory flasks took as usual about 50 minutes. After flasks were placed into the bath they were shaken for 10 minutes. Readings were then started and continued for 3 hours.

As seen from Fig. 2 the course of endogenous respiration after darkening the algal cells depends greatly on the developmental stage of the cells. Small cells

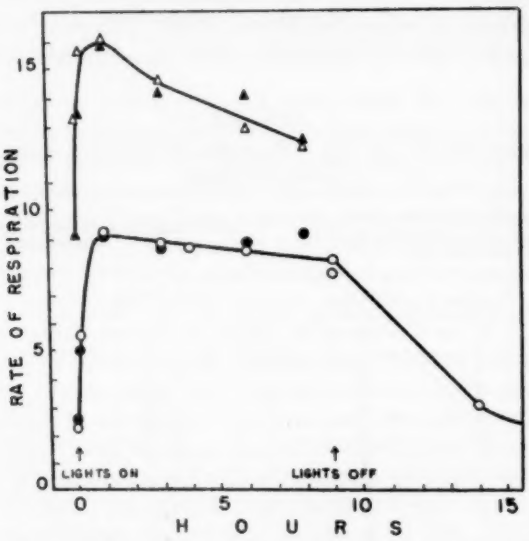

FIG. 1. Rates of endogenous and glucose respiration in cubic millimeters O_2/cubic millimeter packed cells/hour for cells of successive developmental stages of *C. pyrenoidosa*, strain 7-11-05. Open circles, endogenous respiration at pH 4.5; filled circles, endogenous respiration at pH 6.8; open triangles, glucose respiration at pH 6.8, glucose added to the suspending fluid before cells were transferred into it; filled triangles, glucose respiration at pH 6.8, glucose added after 60 minutes of endogenous respiration; all measured by the one vessel method.

from the dark (0 hour cells) have a low respiration rate which decreases only slowly with time. Cells of intermediate developmental stages (1 and 3 hour cells) have the highest starting respiration rate which undergoes a rapid decline as soon as cells are darkened. In cells well advanced in their development (6 and 8 hour cells) the starting respiration rate is lower but this rate remains stable for almost 3 hours of keeping cells in darkness—a surprisingly long time for endogenous respiration, particularly at 39°C. The importance of the time factor in recording respiration rate after harvesting cells is shown in Fig. 3 where the course of respiration during cell development is depicted as based on manometric readings during different intervals of a respiratory experiment.

The longer the preparatory period, and the later the time during which rates of respiration are recorded, the greater is the probability that the curve describing respiration in the cell development will be of the slowly ascending type. In Fig. 3 this would fall somewhere between the curve depicting the rates for 0 to 30 minute interval and that for the 90 to 120 minute interval. It is evident that the course of respiration during cell development, as this process takes place in the growth chamber at the moment of harvesting the cells, is described by neither of these curves but by one more or less approaching that obtained

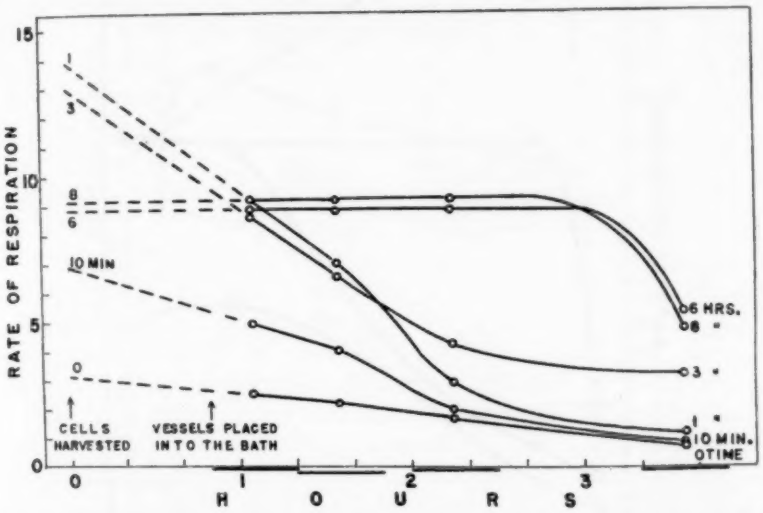

FIG. 2. Time course of the rates of endogenous respiration during respiratory experiment in cubic millimeters O_2/cubic millimeter packed cells/hour for cells of successive developmental stages of *C. pyrenoidosa*, strain 7-11-05. Rates of oxygen consumption calculated for 4 intervals of time, 30 minutes each, indicated on the time scale by the solid lines. The portions of the curves corresponding to the span of time between harvesting the algal cells and placing the manometric vessels into the bath extrapolated graphically. All measured by one vessel method at pH 6.8. Developmental stages are indicated on the curves.

by extrapolation of the data to the moment of harvesting (depicted by the dotted line connecting crosses in Fig. 3). It is interesting that the shape of this extrapolated curve closely approaches that for glucose respiration (Figs. 1 and 4).

Contrary to the picture for endogenous respiration (Fig. 3) the shape of the curve for glucose respiration (Fig. 4) in the course of cell development is far less dependent on the time in which it is recorded during an experiment. The curves in Fig. 4 describing the rate of glucose respiration for two different intervals run almost parallel to each other; the small differences in the shape of these curves will be discussed later.

These studies suggest that the rate of both endogenous and glucose respiration rises immediately after the start of the development of a cell, soon reaches a maximum, undergoes a slow decline, and eventually, by the time the mother

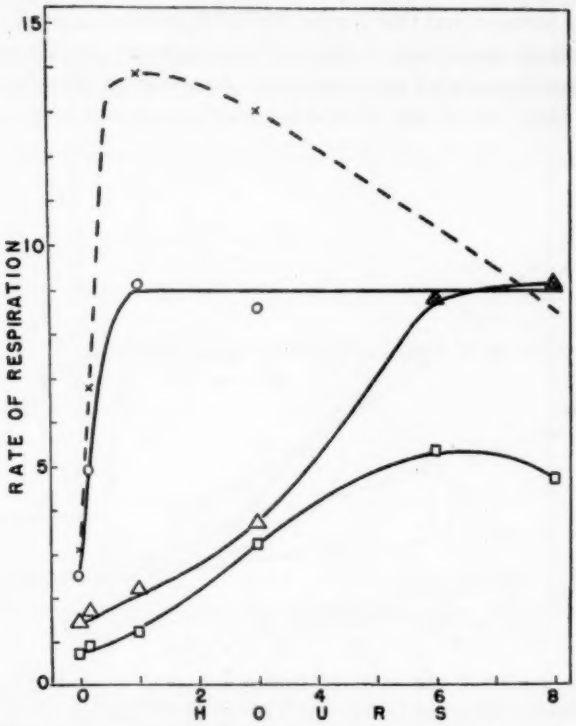

FIG. 3. Rates of endogenous respiration in cubic millimeters O_2/cubic millimeter packed cells/hour for cells of successive developmental stages of *C. pyrenoidosa*, strain 7-11-05. Circles, rates based on readings during the 50 to 80 minute interval after harvesting cells (0 to 30 minute interval after placing vessels into the bath); triangles, rates based on readings during the 140 to 170 minute interval after harvesting cells (90 to 120 minute interval after placing vessels into the bath); squares, rates based on readings during the 200 to 230 minute interval after harvesting cells (150 to 180 minute interval after placing vessels into the bath); crosses, rates extrapolated to the moment of harvesting cells from the growth chamber; measurements by the one vessel method at pH 6.8.

cell ruptures into a number of daughter cells, reaches the lowest level characteristic of autospores in the dark. The dry weight and specific gravity of cells generally increase during development. For this reason the downward slopes of the curves for endogenous and glucose respiration, if calculated on a dry weight basis, become even steeper than in Fig. 1.

Support for this picture of changes in the respiratory rate during the cell development is given by Neeb's data on *Hydrodictyon* (Neeb, 1952), which

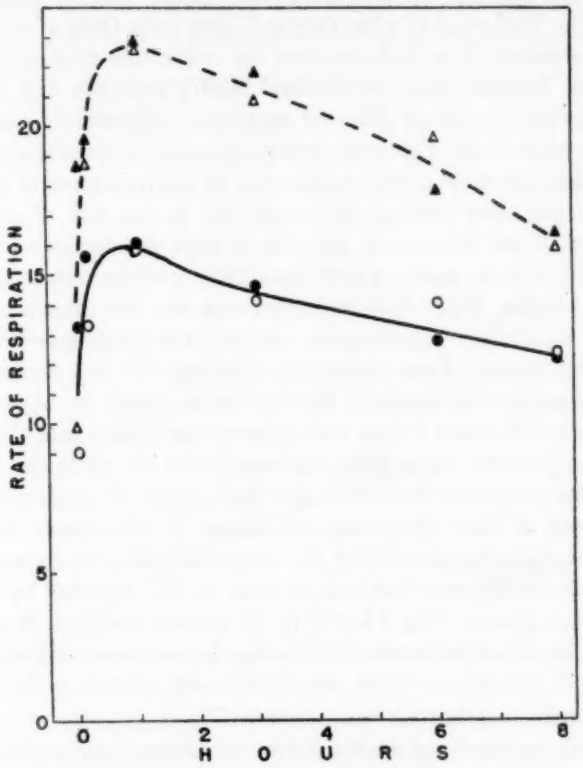

FIG. 4. Rates of glucose respiration in cubic millimeters O_2/cubic millimeter packed cells/hour for cells of successive developmental stages of *C. pyrenoidosa*, strain 7-11-05. Circles, rates based on readings during the 0 to 30 minute interval after placing vessels into the bath or, for the technique in which glucose was added after 60 minutes of endogenous respiration, during the 0 to 30 minute interval after adding glucose; triangles, rates based on readings during the 90 to 120 minute interval after placing vessels into the bath or after adding glucose; filled symbols, glucose added to the suspending fluid before cells were transferred into it; open symbols, glucose added after 60 minutes of endogenous respiration; measurements by the one vessel method at pH 6.8.

show a clearly descending respiration rate during the life cycle of this alga. Neeb did not observe a rise in the respiration rate at the beginning, but he started his respiration measurements 2 days after the start of algal development and possibly missed the phenomenon.

Contrary to these findings Nihei *et al.* (1954) describe the course of the rate of endogenous respiration in *Chlorella ellipsoidea* as gradually rising during the whole period of cell development with the highest value of respiration rate observed at the final stage of what they call light cells. Only after the cell suspension is transferred into darkness does the respiration rate start to decline. Contradictions between these results, and Neeb's and ours may be due to a variety of factors: the use of different organisms, differences in conditions of growing cells, in methods of inducing synchronization, in technique of preparing algal suspensions for manometric studies and in interpretation of the observations. One of the most obvious differences lies in the way of reporting the observed data. What Nihei *et al.* indicate in their discussions as endogenous respiration rate is by no means a rate characteristic of the cells as they grow in the growth chamber. What they measured was the rate after a considerable lapse of time while the cell suspension was prepared for manometric measurements following harvest from the growth chamber. As was shown in Figs. 2 and 3 cells of certain intermediate developmental stages (in our experiments 1 hour, and to a less extent 3 hour cells) possess the highest rate of endogenous and glucose respiration. After being harvested and placed in the dark in the absence of organic carbon these cells use their supply of respiratory material faster than cells of later developmental stages. If manometric readings are delayed long enough after harvesting, the curve describing the course of respiration rate in cell development has a shape close to that reported by Nihei *et al.* (1954) or to that given in Fig. 3 for 90 to 120 minute readings. It can even be concave as in Neeb's experiments (1952) when he measured respiration rate at certain intervals during one light period following a dark period. All these curves are brought together for comparison in Fig. 5.

The time scale in Fig. 5 necessarily differs for different curves. In our experiments it embraces 8 hours, in the observations by Nihei *et al.* it is 28 hours, in Neeb's data for the life cycle of *Hydrodictyon* it is 34 days, and in those depicting the course of respiration during one light period it covers 12 hours. The developmental status of cells to which the starting points of the curves in Fig. 5 refer is also different. In our work the starting point on a curve refers to the zero cells from the dark. In Neeb's experiments, covering the events during one light period, the starting point also indicates cells from the dark. In his experiments, covering the development of the organism for 34 days, measurements were started only after 2 days of growth and therefore the initial period of 2 days has no place on the curve. Nihei *et al.* used a technique of cell synchronization in which a steady state algal suspension grown at *ca.* 6000 lux was transferred to 200 to 400 lux and kept at this light intensity for 7 days. After this time the algal suspension was considered to be rich in what they call "dark" cells and was used as a starting point in their observations on the events taking place during the life history of algal cells. Actually the "dark" cells of

Nihei *et al.* are not comparable in developmental stage to our cells from the dark. After emerging from cell division these cells were immediately influenced by light, and at the start of respiratory measurements they were already in some advanced stage of their development. The absence of the actual zero point in cell

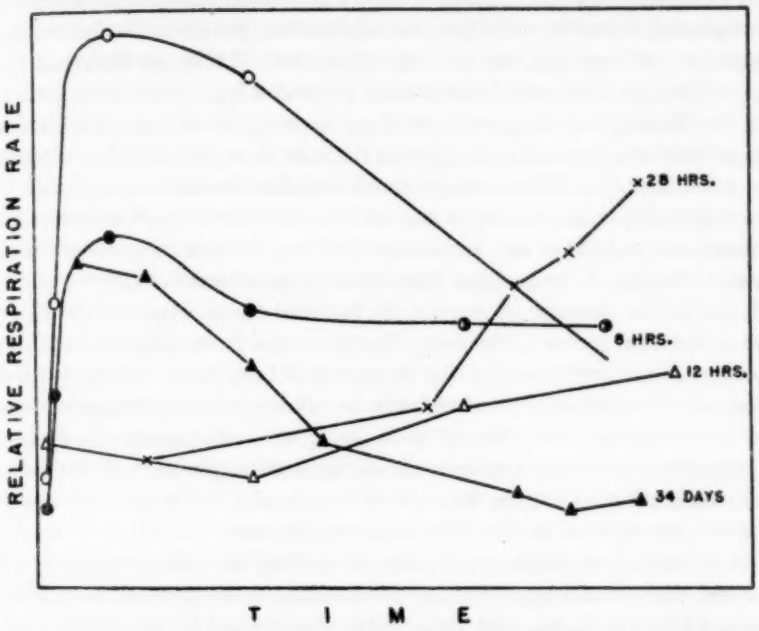

FIG. 5. Rates of endogenous respiration—O_2 consumed—on dry weight basis. Filled circles, data for *C. pyrenoidosa*, strain 7-11-05, for the 8 hour period and based on readings during the 0 to 30 minute interval after placing vessels into the bath; open circles, data for *C. pyrenoidosa* for the 8 hour period extrapolated to the moment of harvesting cells from the growth chamber; filled triangles, Neeb's data for *Hydrodictyon* for the period embracing the course of the development of a colony for 34 days; open triangles, Neeb's data on *Hydrodictyon* for one light period of 12 hours; crosses, Nihei *et al.* data on *C. ellipsoidea* covering the development of a synchronized batch of cells for 28 hours. Time and respiration rate scales are different for different strains.

development from the curve presented by Nihei *et al.* can also help explain the differences in the shape of this curve and ours.

In discussion, it is convenient to distinguish two phenomena: a rapid rise of respiration rate during the first period of cell development and a decline of rate over the second much longer part of the life history of the cell. This decline is slow as long as the algal suspension is kept in the light, but is considerably accelerated by darkening cells at an advanced stage of development.

The slow decline of respiratory rate during development in the light seems

to have no direct connection with the level of carbohydrates in a cell: an increase in the amount of carbohydrates has not been shown to impede respiration. One could think that this slow decline of the rate might be caused by the proportional increase of storage material and dead substance (cell wall) if these processes took place in our experiments during the first 8 hours of cell development. However, this possible explanation proves to be inadequate or insufficient. Microscopic examinations show that, if cells are darkened after 9 hours of illumination, cell divisions take place at a high rate. One might expect that the liberation of daughter cells from mother cell wall and the decrease in proportion of dead material would raise the rate of respiration. This expectation does not materialize. With continued cell divisions the rate of respiration of an algal suspension still declines. It reaches the lowest level at the time when cell divisions are completed and remains at this low level as long as cells are prevented from growth by keeping them dark in the absence of organic carbon.

Probably the decrease in respiratory material plays a part in the rapid decline of respiration rate in the dark. The transition from light to darkness may also directly influence the activity of respiratory enzymes. *But the fact that the decline in the respiration rate starts early in cell development, and extends over the whole period during which the cell is in the light, speaks against the assumption that only these two factors operate to reduce respiration after the cells are darkened.* Evidently bursting of the mother cell wall is preceded by preparatory processes, somehow connected with the decrease in respiration.

The decline of respiration rate after darkening has been studied on steady state suspensions and explained by the decrease in amount of the respiratory substrate (French, Kohn, and Tang, 1934; Cramer and Myers, 1949). The rate of respiration declines very rapidly at first, but after some time (about 25 hours in the French, Kohn, and Tang experiments) comes to an almost steady value, decreasing only slowly thereafter. It was assumed that two substances, A and B, were used in respiration during the first period and the transition to the second period was caused by the exhaustion of A after which only B was used for a long time at a low rate (French *et al.*, 1934).

There is no doubt that a decrease in the amount of respirable material is partially responsible for the decline in endogenous respiration rate with time. But the work with synchronized algal suspensions suggests that the changes in the speed of this decline (the transition from the above mentioned first period to the second) coincide with the accomplishment of cell divisions. The processes bringing about the decline in the respiration rate start early in cell development and culminate at cell division. After that, if cells are prevented from growth by being kept in the dark, the respiration rate decreases very slowly. This course of events with non-synchronized suspensions underlines observations on so called starvation. Darkening a steady state suspension interrupts growth, causes most cells to divide, and this, together with a decrease

in the supply of respirable material, brings the respiration rate rapidly to a low level. After most of the cells are brought to the stage of small autospores their respiration rate changes slowly with time.

The coincidence of cell division with the rapid decline in respiration rate after darkening, can also be demonstrated in another way. It was shown in Fig. 3 that 6 and 8 hour cells have a similar rate of respiration and, if darkened, respire at a constant rate for about 3 hours. One would expect that 6 hour cells which had received less light and therefore accumulated less respirable material would be the first to show signs of a decline in the respiration rate. Actually the opposite is true. The 8 hour cells are advanced further in their development than 6 hour cells and after being darkened, start cell divisions first. The result is that the rate of respiration shows the first signs of a decline in 8 hour cells after 160 minutes in the dark, and at the end of the experiment declines to a lower level than in 6 hour cells in which the respiration rate starts to decline only after 180 minutes in the dark.

To close the cycle of changes in the respiration rate during its life cycle, a cell has to come back to the initial level at the beginning of development. It is difficult to separate the initial rise of rate from the influence of accumulation of carbohydrates in light. The level of carbohydrates in a cell is known to influence the rate of respiration. With the technique of synchronization employed in these investigations the start of cell development and a concomitant rapid rise in respiration rate coincide with the increase in respiratory material in the cell transferred from dark into light. The question is whether this rise in rate is due to anything else than the increase in carbohydrates. An attempt was made to prove that the decline in respiration rate is a normal process taking place during cell development in light. A logical extension of this would be a reverse process bringing the rate of respiration back to a high level to make a new cycle possible.

Another line of reasoning in favor of cyclic changes in respiration rate, induced by one method or another, was provided by the work of Pirson *et al.* (1954) with *Hydrodictyon*. Colonies of this alga were maintained at different regimens of light:dark; *i.e.*, 12:12 hours, 6:6 hours, and 10½ hours:7 hours. Then the conditions were changed to continuous darkness. The rhythmic changes in respiration rate established at each light:dark periodicity continued for some time in the dark with maxima in respiration rate corresponding to the time expected if the particular light:dark regimen had not been changed to continuous darkness. Evidently there was no increase in respiratory substrate after the alga was transferred into darkness; fluctuations in respiration rate occurred with no connection to changes in the level of substrate.

The rate of glucose respiration provides final evidence in favor of the view that increase in respiration rate at the beginning of cell development is influenced by other factors than the level of carbohydrates. As shown in Fig. 4, not

only are the initial rates of glucose respiration different in cells of different developmental stages, but these differences remain after about 2 hours of manometric observations. As a matter of fact these differences become even more pronounced with time.

In the fourth column of Table II the increase in the rates of glucose respiration for 90 to 120 minute intervals of a manometric experiment is expressed in per cent of the rates for 0 to 30 minute intervals. Zero hour cells low in their respiration rate at the start of readings increase the rate by only 9 per cent. Cells in more advanced developmental stages not only have a higher initial respiration rate, but their glucose respiration rate increases more with time, reaching 40 to 47 per cent. In 8 hour cells the rate of glucose respiration is not only comparatively low at the start of an experiment but also increases

TABLE II

Rate of Glucose Respiration in Cubic Millimeters O_2/Cubic Millimeter Packed Cells/Hour for Cells of Successive Developmental Stages of C. pyrenoidosa, Strain 7-11-05

Developmental stage of the cells	Rate of respiration for intervals of time during one respiratory experiment		Per cent increase with time
	0 to 30 min.	90 to 120 min.	
(1)	(2)	(3)	(4)
0	9.0	9.8	9
10 min.	13.3	18.7	41
1 hr.	15.8	22.6	43
3 hrs.	14.1	20.8	47
6 hrs.	14.0	19.6	40
8 hrs.	12.4	15.9	28

more slowly with time (28 per cent) than in cells of earlier developmental stages. Evidently cells of different developmental stages differ in their capacity to respire exogenous carbon sources.

It seems that changes in respiratory activity during cell development are to some extent independent of light. This, however, does not exclude the possibility that a direct light activation of respiratory enzymes takes place parallel to the processes related to cell development. An indirect influence of light through an effect on the respiratory substrate level is also clearly indicated. Studies on the influence of light on respiration have been generally done on steady state suspensions. They have been confused by the fact that changes from light to darkness and *vice versa* have an indirect influence on the rate of respiration by affecting the developmental status of an algal suspension. Large cells which accumulated enough carbohydrates to respire for a considerable length of time at a more or less constant rate, but which did not advance in their development far enough to be ready for cell division, could be given

alternate light and dark periods for some time without influencing their respiration rate. Small daughter cells kept in the dark or under weak illumination can be expected to increase their respiration rate if brought into light or if light intensity is increased. Large cells old enough to divide if darkened may undergo cell division in the course of the experiment and cause the respiration rate to go down. Depending on the previous culture conditions and also on the intensity of illumination and the duration of light and dark periods, the average developmental status of an algal suspension can be changed and a certain degree of synchronization can be induced unknowingly. This will bring about a change in the rate of respiration. Thus the influence of illumination on the rate of respiration of non-synchronized algal suspensions may result from an interplay of a complex array of processes proceeding in different cells at different speed and in different directions. The intricacy of the situation makes it understandable why studies on the influence of light on the respiration of non-synchronized algal suspensions have not produced concordant results.

SUMMARY

Endogenous and glucose respiration were studied during the life history of *Chlorella pyrenoidosa*. A generalized picture of the course of respiration during the life cycle is suggested. At the liberation of daughter cells from the wall of the mother cell, or soon after, the respiration rate reaches its lowest level. If the daughter cells are placed in light the respiration rate rapidly increases with time, soon reaches a maximum, and then declines slowly. Two factors are important in the initial increase—the early developmental stage of the cells and the influence of light. In autotrophically developing algae the parts played by developmental processes and by light have not been separated. Direct activation of respiratory enzymes by light, in addition to the level of respiratory substrate, cannot be excluded.

The decline of respiration rate over most of the cell history seems to have no connection with light and is probably bound to the developmental processes *per se*. Darkening the suspension interrupts growth and induces liberation of daughter cells, with concomitant faster decrease in respiration rate. The rate of respiration of small daughter cells decreases in darkness only slowly with time. Illumination seems necessary to bring these cells back to a high level of respiratory activity.

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THE EQUILIBRIUM BETWEEN CYTOCHROME OXIDASE AND CARBON MONOXIDE

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(Received for publication, August 3, 1956)

Hemoglobin occurs in the bloods of all vertebrates except a few fishes. In invertebrates, however, it appears only sporadically, cropping up in isolated species whose closest relatives often lack this pigment. Indeed here it is distributed so haphazardly as for a time to have discouraged the hope that one might ever discover lines of biochemical relationship among animals comparable with their lines of anatomical relationship. This situation was not improved by the discovery of hemoglobins in Protozoa (Sato and Tamiya, 1937; Keilin and Ryley, 1953); in the root nodules of leguminous plants (Keilin and Wang, 1945); and in a number of molds (Keilin, 1953; Keilin and Tissières, 1953).

With the realization, however, that hemoglobin is closely related chemically to the heme enzymes of cellular respiration—cytochrome oxidase, the cytochromes, catalase, and the peroxidases—this situation became more understandable. Such enzymes occur in all aerobic cells, and it seems probable that in the course of evolution hemoglobins have repeatedly been derived from them (Barcroft, 1928, chapter 4; Anson and Mirsky, 1930, p. 525).[1]

In an attempt to define this development more closely it has been suggested that the hemoglobins evolved in three stages, involving three fundamental properties (Wald, 1952). The stages were: (1) Heme enzymes, among which one thinks first of cytochrome oxidase, the only member of this class that unites directly with oxygen and carbon monoxide. (2) Storage hemoglobins, concerned primarily with *holding* oxygen; *i.e.*, with increasing the oxygen *capacity* of the cells and tissues. (3) Circulatory hemoglobins, concerned primarily with *transporting* oxygen. The properties of hemoglobin that alter progressively in this development are: (1) The affinity for oxygen decreases. (2) The shape of the oxygen equilibrium curve—percentage oxygenation *vs.* oxygen pressure—changes from a rectangular hyperbola to a more and more sharply inflected S.

* This investigation was supported in part by funds from The Rockefeller Foundation and the Office of Naval Research.

[1] Compare Anson and Mirsky (1930, p. 525): "Hemoglobin, it now appears, may no longer be regarded as the pre-eminent heme pigment, nor is its haphazard distribution now so mysterious. Hemoglobin is merely an occasional specialized derivative of an iron substance, heme, which is much more widely distributed."

(3) The affinity for oxygen declines more and more sharply with rise in acidity: the Bohr effect.

This development is cumulative; vertebrates in general possess all its stages: cytochrome oxidase in all the cells; the typical storage hemoglobin, myoglobin, in the muscles; a circulatory hemoglobin in the blood. Indeed a single organ such as the beef heart, from which we derived our cytochrome oxidase, displays all these stages.

Muscle and blood hemoglobins are already known to be related in their properties as here described. Muscle hemoglobin has a relatively high affinity for oxygen, a hyperbolic oxygen equilibrium curve, and a very small Bohr effect; blood hemoglobin a much lower oxygen affinity, an S-shaped equilibrium curve, and a relatively large Bohr effect.

Of cytochrome oxidase in this regard one knows only that it possesses a relatively high affinity for oxygen. The evolution argument reviewed above implies that it should also possess an approximately hyperbolic equilibrium function, and little if any Bohr effect. The object of the present investigation is to inquire into these properties.

The reaction of oxygen with cytochrome oxidase, however, is quite different from that with hemoglobin. The former is a true oxidation, changing the iron of cytochrome oxidase from the ferrous to the ferric condition; whereas the reaction with hemoglobin is only an "oxygenation" which leaves the iron ferrous as before. Cytochrome oxidase does not enter into an equilibrium with oxygen, but is oxidized by it.

For this reason we studied instead the reaction between cytochrome oxidase and carbon monoxide. This is a true equilibrium, strictly analogous with that between carbon monoxide or oxygen and hemoglobin. In all hemoglobins so far examined, though the affinities for oxygen and carbon monoxide may differ widely, the shape of the equilibrium function and the vigor of the Bohr effect appear to be the same with both gases (*cf.* Barcroft, 1928 pp. 148–160). For these two properties therefore the reaction with carbon monoxide provides a reliable analogy to that with oxygen.

In a sense therefore, in these experiments, we examine cytochrome oxidase as though it were a hemoglobin. Nevertheless our observations bear also upon its general properties, as an enzyme and a heme protein.

Methods

Preparation.—The beef heart preparation of Ball and Cooper (1949) was used as the source of cytochrome oxidase. The ammonium sulfate precipitate was suspended in distilled water, and centrifuged for 1 hour at 5°C., at 25,000 R.P.M. (41,200 *g*) in the Spinco preparative ultracentrifuge. The clear, colorless supernatant was discarded. The brown pellet was transferred to a drying tube, frozen in a bath of dry ice in acetone, and lyophilized. The resulting powder was extracted twice with pe-

troleum ether. The last traces of petroleum ether were removed under vacuum, and the powder stored at −20°C. until required.

To dissolve the cytochrome components, 0.120 gm. of the dried powder was ground in a Potter-Elvehjem homogenizer with 4 ml. of 2 per cent sodium desoxycholate (L. Light and Co.) in M/20 glycylglycine buffer at pH 8.3. The resulting suspension was centrifuged for an hour at 25,000 R.P.M. in the Spinco ultracentrifuge. The clear brown supernatant was decanted and used in our experiments. Its pH was about 7.4.

Carbon Monoxide Equilibrium.—The tonometer was similar to that described by Allen, Guthe, and Wyman (1950), but adapted to fit into the Beckman spectrophotometer. The opening for injection of liquids and gases had been moved to the side of the equilibration chamber. The optical path was 1 cm.

The sodium desoxycholate solution of cytochrome components (3 ml.) was pipetted into the tonometer, and the latter sealed with a rubber vaccine cap. Air was removed by successive evacuations through the stopcock and by filling with nitrogen. Evacuation was by water aspirator, with the tonometer immersed in cracked ice to prevent foaming. The solution in the tonometer was then equilibrated by rotation in a water bath for 15 minutes.

During this time a few milliliters of 2 per cent desoxycholate solution in glycylglycine buffer was washed with nitrogen to remove oxygen.

To reduce the cytochrome system, about 10 mg. of sodium dithionite ($Na_2S_2O_4$) was placed in a dry syringe, and 1 ml. of the sodium desoxycholate solution under nitrogen drawn into it. 0.5 ml. of the resulting solution was injected immediately into the tonometer through the vaccine cap. This brings the pH of the cytochrome preparation to about 7 unless alkali is added to the buffered desoxycholate used to dissolve the dithionite. The tonometer was again evacuated and rinsed with nitrogen, usually three times. Finally it was equilibrated for 20 minutes under vacuum.

Nitrogen and carbon monoxide were mixed at constant volume, in proportions determined by measuring the pressure with a mercury manometer. The mixtures were stored in a bottle under positive pressure. The bottle had five outlets, sealed with rubber vaccine caps, from which gas could be taken with a calibrated hypodermic syringe. The syringe was rinsed three times with the gas mixture, then filled and a measured amount of the gas injected into the tonometer through the rubber cap. The tonometer was then rotated for 20 minutes in a water bath at a temperature of 10° ± 0.1°C. Tests showed this period to be sufficient for attaining equilibrium. Further details of these procedures have been described by Allen *et al.* (1950) and by Riggs (1951–52).

After equilibration with each pressure of carbon monoxide, the extinction of the preparation was measured at 445 and 650 mμ. These measurements were made in a cold room kept at 10° ± 2°C. After such determinations had been completed at five different pressures of CO, the tonometer was filled with pure CO and reequilibrated for 20 minutes. Then the entire absorption spectrum was measured from 440 to 650 mμ.

Finally the tonometer was allowed to warm to room temperature and the pH of the preparation determined with a Beckman pH meter, immediately on removal from the tonometer.

Enzyme Activity.—This was measured by the method of Potter (*cf.* Umbreit *et al.*,

1949). The preparation, to which cytochrome *c* in phosphate buffer and dilute aluminum chloride had been added, was placed in the main chamber of a Warburg manometer, with sodium ascorbate in the side compartment. After thermal equilibrium was established, the ascorbate was tipped into the main chamber, and the oxygen uptake measured. The solution was then stored overnight in the refrigerator, and its absorption spectrum measured in the oxidized state, reduced, and combined with CO.

Observations

Absorption Spectra.—The absorption spectra of all our preparations were essentially identical. Fig. 1 shows a representative series, all at equivalent concentration: the preparation in air (oxidized), reduced with dithionite, and treated with carbon monoxide. Below 480 mμ the extinctions rise rapidly, and the continuation of the spectra in this region is shown on a more compressed scale in Fig. 2. The difference spectrum of the carbon monoxide complex—the difference in absorption spectrum between it and the reduced preparation—is shown in Fig. 3.

These spectra are very similar to those published by Ball, Strittmatter, and Cooper (1951) on a similar preparation. Since our experiments were completed (1952), such preparations have been resolved further, so as to yield essentially mixtures of cytochrome oxidase and cytochrome *a* (Smith and Stotz, 1954; Smith, 1955*a*,*b*; Wainio, 1955). Our preparation contained in addition cytochrome *b* and some *c*; but there is no reason to believe that this should have influenced the results of our measurements.

The oxidized preparation displays little more than absorption rising continuously into the ultraviolet, owing probably in large part to light scattering. Superimposed on this is a small absorption peak at about 525 mμ and a small inflection at about 590 mμ. On reduction the bands of the cytochromes appear: *a* at about 605 mμ, *b* and *c* at 552 to 562, and a band at 525 mμ attributable to all three cytochromes.

On exposure to CO, small changes occur in most parts of the spectrum. These appear to involve specifically cytochrome oxidase. The only contaminants that would react with CO are blood and muscle hemoglobins; and we believe these to be virtually absent from our preparations for the following reasons: (*a*) washings of our preparations with distilled water are colorless; (*b*) the oxidized preparations show no trace of the oxyhemoglobin peaks at 540 and 570 mμ; and (*c*) the preparations exposed to CO show no sign of the shift of absorption toward the red below 650 mμ that would accompany the formation of CO-hemoglobin (*cf.* Horecker, 1943). On the contrary the CO-complex formed with our preparations displays the lowest absorption at these wave lengths (Fig. 1).

The difference spectrum shown in Fig. 3 therefore appears to represent cytochrome oxidase. It is almost identical with that obtained by Ball, Strittmatter,

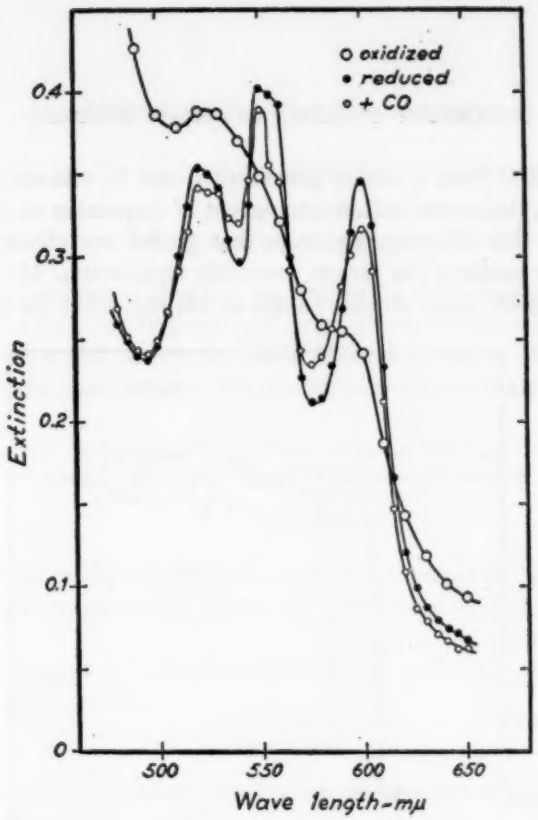

FIG. 1. Absorption spectra of a preparation of cytochrome system components of beef heart in sodium desoxycholate solution: oxidized in air; reduced with dithionite; and saturated with carbon monoxide. pH 7.60. The absorption bands in the reduced preparation display the presence of cytochromes *a* and *b*, and a small amount of *c*. The changes in spectrum caused by carbon monoxide appear to be due entirely to cytochrome oxidase.

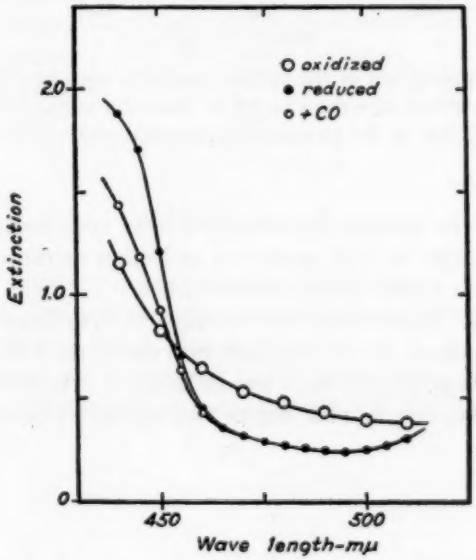

FIG. 2. Extension of the spectra of Fig. 1 to lower wavelengths, plotted on a compressed scale of extinctions.

and Cooper (1951) from a similar preparation, and by Chance and Williams (1955) for the cytochrome oxidase component of suspension of rat liver mitochondria. It is this difference spectrum that guided our choice of the wave lengths used to measure the carbon monoxide equilibrium: 445 and 650 mμ. Our calculations are based on the changes at 445 mμ; while the measurements

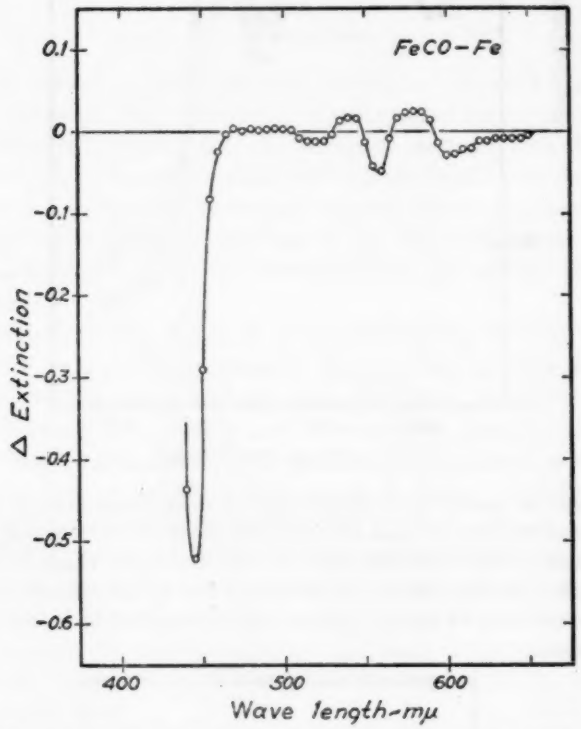

FIG. 3. Difference spectrum of the carbon monoxide complex of beef heart cytochrome oxidase. To obtain this the absorption spectrum of the reduced preparation was subtracted from that of the preparation saturated with carbon monoxide (Figs. 1 and 2).

at 650 mμ, where no changes are expected from cytochrome oxidase, were used to control changes in light scattering and other extraneous disturbances that might affect the course of the measurements.

Carbon Monoxide Equilibrium.—Presumably in cytochrome oxidase as in hemoglobin, one molecule of CO combines with one atom of Fe. We do not yet know how many Fe-porphyrin units one molecule of cytochrome oxidase contains; or if more than one, to what degree they interact with one another. The

general equation for the combination of cytochrome oxidase with CO may be written:

$$Fe_n + n\ CO = (FeCO)_n$$

in which n is an empirical constant, not necessarily an integer, which represents the degree to which the Fe-porphyrin units of cytochrome oxidase interact in their combination with carbon monoxide. If the enzyme contains only one such unit per molecule, or if the units combine with CO independently of one another, n=1.

The corresponding mass action equation can be written:

$$K = \frac{[(FeCO)_n]}{[Fe_n]pCO^n}$$

in which pCO is the partial pressure of CO in the gas phase, and K, the equilibrium constant, measures the affinity of cytochrome oxidase for carbon monoxide.

When the enzyme is half-saturated with CO, pCO may be written as p_{50}; and since $(FeCO)_n = Fe_n$,

$$p_{50}^n = 1/K$$

The constant p_{50} is therefore a useful inverse measure of the affinity, and a means of evaluating K.

For some purposes these relationships are expressed more conveniently in Hill's equation:

$$\frac{y}{100} = \frac{Kp^n}{1 + Kp^n}$$

in which y is the percentage saturation, p the CO pressure, and K and n have their earlier meanings. When $n = 1$, this is the equation of a rectangular hyperbola; when n is greater than 1, the curve is S-shaped.

To evaluate n it is most convenient to throw this and the preceding equation into logarithmic form:

$$\log K = -n \log p_{50}$$

$$n \log \frac{p}{p_{50}} = \log \frac{y}{100 - y}$$

This last is the equation of a straight line of slope n.

The results of five experiments made with two different preparations are summarized in Table I and in Figs. 4 and 5.

In Fig. 4 all the data are plotted in the conventional way, that of the Hill equation. p_{50} in all the experiments lies between 0.16 and 0.18 mm.; the average

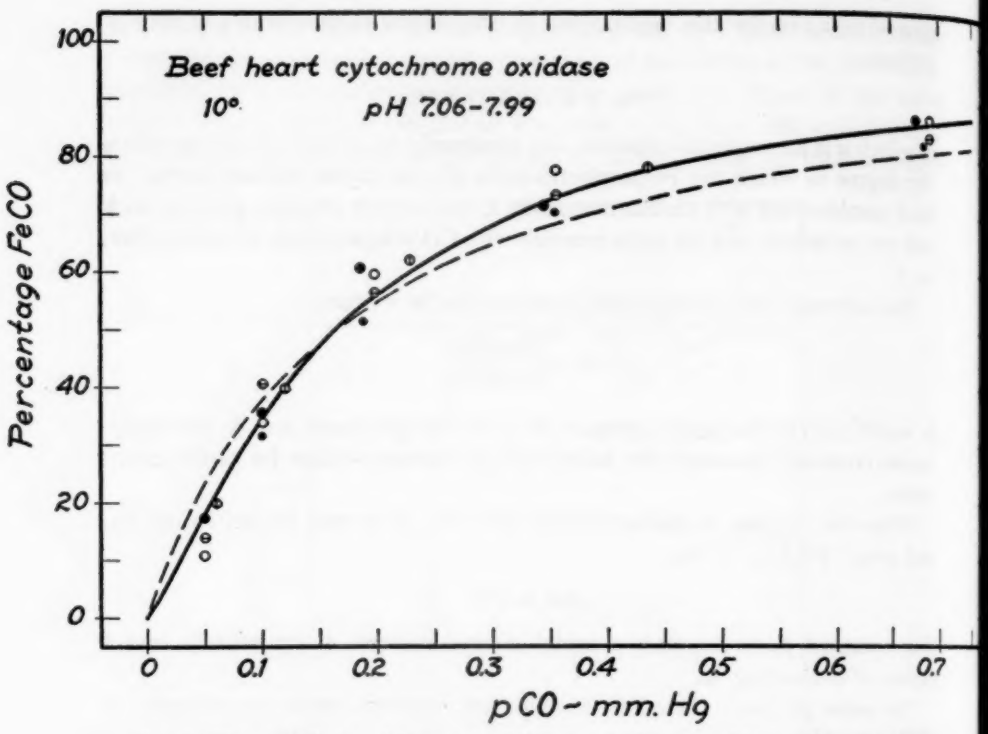

FIG. 4. The equilibrium between beef heart cytochrome oxidase and carbon monoxide: percentage saturation (y) plotted against partial pressure of carbon monoxide (p). Data of five experiments involving two different preparations. The solid line is drawn according to the Hill equation, $y/100 = 9.57\ p^{1.26}/(1 + 9.57\ p^{1.26})$. The broken line shows the corresponding rectangular hyperbola, in which the exponent of the above equation has been changed from 1.26 to 1.

TABLE I

Data of five experiments performed with two different preparations of beef heart cytochrome system components dissolved in 2 per cent sodium desoxycholate, and buffered with glycylglycine. p_{50} is the partial pressure of carbon monoxide at which the cytochrome oxidase is 50 per cent saturated; n is the exponent in the Hill equation. All preparations equilibrated at 9.8° ± 0.1° C.

Preparation	pH	p_{50} (mm. Hg)	n
I	7.06	0.162	1.36
II	7.60	0.178	1.26
	7.84	0.182	1.26
	7.38	0.148	1.27
	7.99	0.155	1.26
	Averages	0.165	1.28

value is 0.165 mm. (Table I). This way of plotting the data suggests the presence of a slight inflection; and this is confirmed by the logarithmic plot of the same data in Fig. 5. The slope of the line in Fig. 5 is $n=1.26$; the value $n=1$

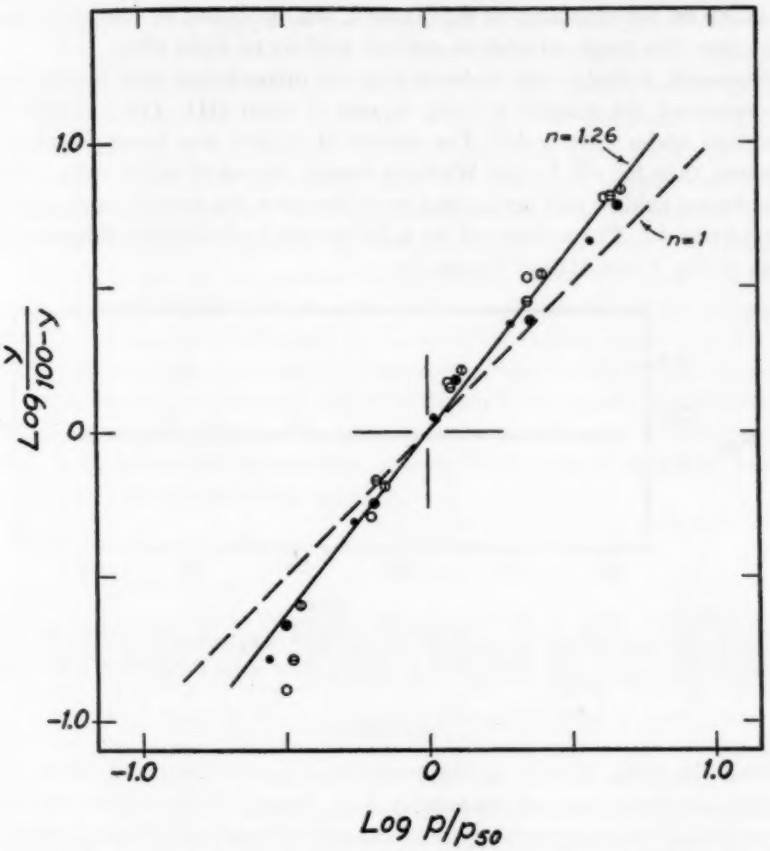

FIG. 5. The data of Fig. 4 replotted in logarithmic form, so as to yield a straight line of slope n. The best fit of these data shows that $n = 1.26$. The line for $n = 1$, implying that the oxygen equilibrium curve is a rectangular hyperbola, clearly is inadequate.

clearly is inadequate. Using $n = 1.26$ and $p_{50} = 0.165$, $K = 9.57$, and the Hill equation for these data is:

$$\frac{y}{100} = \frac{9.57p^{1.26}}{1 + 9.57p^{1.26}}$$

This equation was used to draw the solid curve in Fig. 4. The corresponding rectangular hyperbola ($n = 1$; other constants as before) is drawn in this

figure with a broken line. The rectangular hyperbola fails by very little, yet significantly, to describe the data.

Bohr Effect.—The above measurements were made at pH's varying between 7.06 and 7.99. Over this range the affinity for CO does not change significantly, as shown by the constancy of p_{50} (Table I, Fig. 6). It can be concluded that at least over this range cytochrome oxidase exhibits no Bohr effect.

Enzymatic Activity.—As evidence that our preparations were in good order, we measured the enzyme activity of one of them (II). The procedure was described under "Methods." The uptake of oxygen was linear for about 35 minutes, then fell off. In two Warburg vessels, to one of which twice as much cytochrome oxidase had been added as to the other, the ratio of rates of oxygen uptake was 1.7. The activity of the solution which yielded the difference spectrum of Fig. 3 was 3410 μl. O_2/ml./hr.

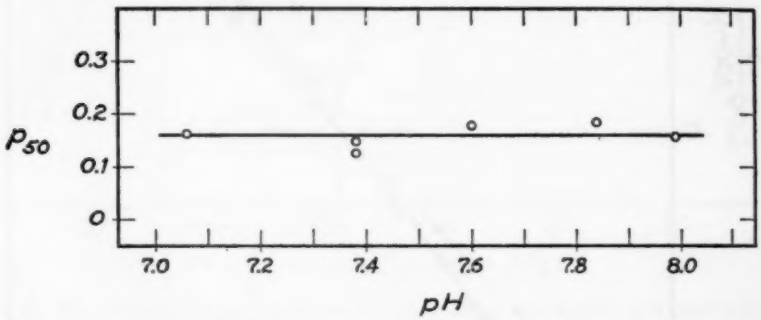

FIG. 6. The variation of p_{50} with pH. The affinity of cytochrome oxidase for carbon monoxide does not change from pH 7 to 8; *i.e.*, there is no Bohr effect in this range.

DISCUSSION

From the point of view of the evolution argument reviewed above, these experiments have come out reasonably well. Among the properties with which the argument was concerned, cytochrome oxidase takes an extreme position in two, and nearly so in the third. In affinity for oxygen it was already known to exceed all the hemoglobins with which it is associated. We can now add that it lacks the Bohr effect, as does no known hemoglobin. In the usual mammalian blood hemoglobin, p_{50} is increased 3 to 4 times by a fall of pH from 8 to 7; in horse muscle hemoglobin it rises about 25 per cent (Theorell, 1934); in beef cytochrome oxidase it remains unchanged. In the third property, the shape of the equilibrium function, cytochrome oxidase has nearly the extreme hyperbolic form, though departing from this measurably.

All such evolutionary arguments are no more than orderly arrangements of data, fitting what would otherwise be unrelated facts into the context of a larger construction. At best one hopes to derive from them some stimulus for

further inquiry, and some measure of prediction of further observations. The present argument can be said to be serving these purposes.

What justification exists for transposing information gained with carbon monoxide to the combination of cytochrome oxidase with oxygen? First, as to hemoglobins: with regard to the properties we have examined—the interaction of Fe-porphyrin groups and the Bohr effect—hemoglobins display an extraordinary identity of behavior toward oxygen and carbon monoxide (Barcroft, 1928, pp. 148–160). One would like to assume that some such parallelism holds also for cytochrome oxidase; but here one must meet the problem that this enzyme reacts quite differently with both gases. As already noted, with carbon monoxide it enters a true equilibrium, whereas with oxygen it undergoes an intrinsically irreversible oxidoreduction. How then compare them?

The point is that in the presence of suitable reducing systems, cytochrome oxidase is continuously reduced, and continuously reoxidized by oxygen. If only a limited amount of reductant is available, the rate of reduction will fall off with its concentration. More usually, however, reductants are present in excess, and in this condition the rate of reduction depends only upon the concentration of oxidized enzyme. The enzyme then enters a steady state or pseudoequilibrium, in which its rate of reduction is equal to its rate of oxidation. For the steady state one can write the equation:

$$K' = \frac{[(FeO_2)_n]}{[Fe]_n p{O_2}^n}$$

in which Fe represents the Fe-porphyrin groups—one or more—which combine with a single molecule of oxygen; and n represents the degree of interaction among the oxygen-combining centers. That is, such a steady state equation has exactly the same form as the equation for the equilibrium of cytochrome oxidase with carbon monoxide, or of hemoglobin with either gas (*cf.* Warburg, 1927). In the presence of excess reductant, the pseudoequilibrium between cytochrome oxidase and oxygen can be treated just as are these true equilibria.

From this point of view the properties we have considered probably have considerable importance in the physiological functioning of cytochrome oxidase. The business of this enzyme is to make oxygen available for cellular respiration. Its high affinity enables it to take oxygen away from blood and tissue hemoglobins, and to maintain maximal rates of respiration down to very low oxygen pressures. The nearly hyperbolic form of its (pseudo-) equilibrium curve—which we assume, as in the hemoglobins, to be much the same with oxygen as with carbon monoxide—facilitates a high degree of saturation at low oxygen pressures. The absence of Bohr effect—which again as in the hemoglobins we assume to be the same with oxygen as with carbon monoxide—means that in conditions involving oxygen debt, as in anoxia or violent work, as the cell grows more acid, this change does not interfere with its respiration.

In these properties cytochrome oxidase lies at the other extreme from a circulatory hemoglobin. The business of the latter is not only to combine with oxygen in the gills or lungs, but to discharge it in the tissues, a function promoted by just those properties cytochrome oxidase lacks: a low affinity for for oxygen, a strongly inflected S-shaped equilibrium curve, and a large Bohr effect.

The pressures of carbon monoxide at which our preparations were half-saturated—0.16 to 0.18 mm.—seem surprisingly low. In part this is the result of the low temperatures at which these measurements were made. If we can assume as with hemoglobin that p_{50} rises about 3 times for each 10° rise in temperature (*cf.* Barcroft, 1928, p. 174), the p_{50} should be about 3 mm. at 37.5°C. In recent measurements with a beef heart particle system at pH 7.37 and 37.7°C., Ball *et al.* (1951) found the oxidation of two substrates to attain half the maximal rate at about 10 mm. oxygen. This represents the p_{50} of the cytochrome oxidase–oxygen pseudoequilibrium *under these conditions of reduction.*

These measurements make it appear that cytochrome oxidase has a higher affinity for carbon monoxide than for oxygen; yet just the contrary is commonly stated to be true. When intact cells or cytochrome preparations are exposed to mixtures of carbon monoxide and oxygen, and rates of respiration or oxidation are measured, it appears that to inhibit such rates 50 per cent requires that the gases be in the ratio $CO/O_2 = 2.2 - 28$ (Warburg, 1926; *cf.* review of such data by Ball *et al.*, 1951). These values have somewhat the force of p_{50} ratios for the two gases in their combination with cytochrome oxidase, and imply that oxygen has much the greater affinity.

As already remarked, however, though the reaction with carbon monoxide is a simple, reversible addition, yielding a true equilibrium, the reaction with oxygen is an intrinsically irreversible oxidation, yielding at most a pseudo-equilibrium or steady state in the presence of excess reductant. (In the latter instance such a term as "affinity" has only an arbitrary meaning; the p_{50} depends directly upon the velocity of reduction.) This distinction lends oxygen a great advantage when both gases are placed in competition. It is probably true, as our measurements show, that carbon monoxide, when not competing with oxygen, has a higher affinity for cytochrome oxidase than had been supposed; yet for the reason given it is only a feeble respiratory poison.

The departure of the carbon monoxide equilibrium curve from a hyperbola is so small as to have little physiological effect in itself; yet it carries the important implication that since the Fe-porphyrin groups of cytochrome oxidase interact to a degree, this enzyme must contain *more than one Fe-porphyrin unit per molecule.*

The interaction raises a further consideration. The reaction of cytochrome oxidase with oxygen involves both combination and oxidation, and so could

include two distinct types of interaction: (1) the combination of the enzyme with a first molecule of oxygen could facilitate the binding of a second molecule of oxygen; and (2) Fe-porphyrin units could cooperate in reducing oxygen.

Much has been made recently of the thought that the chain of respiratory processes passes from systems concerned with two-electron transport (DPN and flavine enzymes) to others concerned with one-electron transport (metalloenzymes, including those of the cytochrome system). It should not be forgotten, however, that at the end of the chain lies the diatomic molecule, O_2, which requires *two* electrons for its reduction. The passage of one electron to oxygen yields the superoxide ion, O_2^-, or in water the radical HO_2, both highly unstable in the free state; whereas two electrons form the stable peroxide ion, $O_2^=$, or in water H_2O_2.

If the Fe-porphyrin groups of cytochrome oxidase united with oxygen in the same proportion as in the hemoglobins ($Fe^{++}:O_2$), only one electron could be transmitted at a time. One would have to assume a reduction of oxygen in two stages, passing over the unstable intermediate O_2^-. Such a sequence of reactions presents obvious kinetic and thermodynamic difficulties.[2]

It is intrinsically more probable that in cytochrome oxidase *two* Fe-porphyrin units combine with one molecule of oxygen in the manner Fe^{++}–O_2–Fe^{++}. In this case two electrons could pass almost simultaneously from iron to oxygen, yielding two ferric ions and releasing the oxygen as hydrogen peroxide. Such an arrangement should bring cytochrome oxidase into close relationship with hemocyanin, in which copper unites with oxygen in the proportion 2 $Cu^+:O_2$, with the resultant oxidation of one or both cuprous ions (Schulman and Wald, 1951; Klotz and Klotz, 1955); and with hemerythrin, in which iron appears to combine with oxygen in the proportion 2 $Fe^{++}:O_2$, with the consequent oxidation of both ferrous ions (Klotz and Klotz, 1955).

On the other hand it is unlikely that carbon monoxide can form a similar bridge between two iron atoms. In cytochrome oxidase iron probably combines with carbon monoxide in the same proportion as in the hemoglobins, $Fe^{++}:CO$. It is possible therefore that in this enzyme two Fe-porphyrin units that facilitate each other in combining with *two* molecules of CO, combine with *one* molecule of oxygen. In that case the equilibrium curve which we found slightly inflected with carbon monoxide ($n = 1.26$) might become a rectangular hyperbola with oxygen ($n = 1$).[3] Our observations, therefore, need not be in conflict

[2] This discussion owes much to Michaelis (1948), who remarked upon the almost unique peculiarity of hemoglobin in combining ferrous ions with oxygen without oxidation, and went on to say, "One may wonder whether the function of—globin in hemoglobin is to fix the position of the heme molecules—so that an oxygen molecule attached to one of them can never form a bridge to another."

[3] It is interesting that reduced alkali-denatured hemoglobin ("globin-hemochromogen") yields a hyperbolic equilibrium function with carbon monoxide. (Anson and

with the report of Ludwig and Kuby (1955) that the percentage oxidation of cytochrome oxidase rises hyperbolically with oxygen concentration in living yeast.

These considerations yield some insight into the possible intramolecular mechanism of the evolution of hemoglobins from cytochrome oxidase. Let us assume, pending further examination, that in cytochrome oxidase two Fe-porphyrin units combine with one molecule of oxygen in the arrangement Fe^{++}-O_2-Fe^{++}, so facilitating the oxidoreduction that is the principal business of this enzyme. The first step in forming a hemoglobin would involve separating the Fe-porphyrin units, so that each combines independently with oxygen (Fe^{++}-O_2) (*cf.* Michaelis, 1948.). The iron would then tend to remain ferrous; oxidation would give way to oxygenation. A further consequence of separating the hemes would be the almost complete loss of interaction; the equilibrium curve would be close to hyperbolic, n nearly equal to 1. These are the properties of the typical storage hemoglobins of muscle and most invertebrate bloods. Finally, a new mechanism of interaction among the hemes is evolved, which, while still preventing the formation of an oxygen bridge between two iron atoms, mutually facilitates the combination with oxygen. This has given us the typical circulatory hemoglobins of vertebrate blood, with strongly inflected oxygen equilibrium curves (n = 2 to 3). The mode of attachment of heme groups to globin holds them apart sufficiently so that an oxygen molecule can unite with only one. The interaction is transmitted through the protein, and apparently involves sulfhydryl groups (Riggs, 1952–53; Riggs and Wolbach, 1955–56).

It is necessary only to denature oxyhemoglobin to have it oxidize rapidly to methemoglobin (Anson and Mirsky, 1925). Possibly the disorganization of the globin structure permits Fe-porphyrin units to come together, and so co-operate in passing electrons to oxygen, as we suppose happens normally in cytochrome oxidase.

SUMMARY

An evolution argument which attempted to trace the development of hemoglobins from such respiratory pigments as cytochrome oxidase presupposed that the latter possesses, in addition to its high affinity for oxygen, an approxi-

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Mirsky, 1925). The denaturation of globin abolishes the interaction among the hemes, so that each apparently combines independently with CO. "Haemoglobin is not merely an organic compound perhaps a bit more complicated than the rest. It is a biological molecule. It has structure, just as an organism has structure. It has organisation, just as an organism has organisation. Destroy that structure. What is left has the same chemical composition as at the start. But all the biologically valuable qualities have disappeared. Haemochromogen, despite its iron and its pyrrol and its protein, is dead and useless" (Anson and Mirsky, 1925).

mately hyperbolic equilibrium function, and little if any Bohr effect (decline in affinity for oxygen with rise in acidity). Since cytochrome oxidase, unlike hemoglobin, is irreversibly oxidized by oxygen, the present experiments examine its combination with carbon monoxide, with which, like hemoglobin, it yields a true equilibrium. In all known hemoglobins the form of the equilibrium function and the vigor of the Bohr effect are similar with carbon monoxide and with oxygen, so that observations involving the former gas are relevant to the relations of the latter.

The equilibrium function of cytochrome oxidase with carbon monoxide—percentage saturation *vs.* partial pressure of CO—is slightly inflected (in the Hill equation $n = 1.26$; for a hyperbola, $n = 1$). No Bohr effect is present in the range of pH 7–8. The pressure of carbon monoxide at which half-saturation occurs (p_{50}) is about 0.17 mm. at 10–13°C. The affinity for carbon monoxide is therefore higher than commonly supposed.

These properties are consistent with the evolution argument. They are important also for the physiological functioning of cytochrome oxidase, the nearly hyperbolic equilibrium function facilitating a high degree of saturation, and the lack of Bohr effect making this enzyme impervious to hyperacidity.

The slight inflection of the equilibrium function shows that the Fe-porphyrin units of cytochrome oxidase interact to a degree, hence that the enzyme must contain more than one such unit per molecule. It is suggested that in cytochrome oxidase two Fe-porphyrin groups may unite with one oxygen in the manner Fe^{++}–O_2–Fe^{++}; and that the evolution of hemoglobins proceeded over a first stage in which the hemes were separated so that each combines with only one molecule of oxygen, so tending to remain reduced; to a further stage in which the separated hemes interact through the protein to facilitate one another in combining with oxygen.

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THE VITAMIN A OF THE LOBSTER

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(Received for publication, September 27, 1956)

Vitamin A appears to play much the same role in the vision of many invertebrates as it does in the vertebrate eye. In the squid, *Loligo pealii*, the retina contains vitamin A and a high concentration of retinene, the latter mostly bound in the visual pigment rhodopsin (Wald, 1941; Bliss, 1948; St. George and Wald, 1949). The eyes of the fresh water crayfish, *Cambarus virilis*, contain both vitamin A and retinene as well as astaxanthin. Those of marine decapod crustacea—green and fiddler crabs, lobsters—contain high concentrations of vitamin A in addition to astaxanthin and other carotenoids found in the integument (Wald, 1941, 1943, 1945–46). Recently Fisher, Kon, and Thompson (1952, 1954, 1955) have found particularly large amounts of vitamin A in the eyes of euphausiid crustacea.[1]

It is a remarkable fact that these animals contain little or no vitamin A in their bodies. In the squid no trace of vitamin A or other carotenoid could be found in the whole bodies less the eyes (Wald, 1941).[2] More than 90 per cent of the total vitamin A in euphausiid crustacea is found in their eyes. Frequently vitamin A could not be identified at all in other tissues, or in whole bodies less the eyes. The same was true of a number of decapods: lobsters, green crabs, and shrimp (Fisher *et al.*, 1952, 1955).[3]

* This investigation was supported in part by funds from The Rockefeller Foundation and the Office of Naval Research. An account of it was presented at the meeting of the Society of Biological Chemists in San Francisco, April, 1955 (Wald and Burg, 1955).

[1] Lönnberg (1935, 1936) has reported the presence of carotenoids in extracts of crustacean and octopus eyes. Positive antimony chloride tests were obtained from these extracts, but vitamin A was not explicitly identified.

[2] Fox and Crane (1942) found no carotenoids in the Pacific squid, *Loligo opalescens*; but did find them in the octopus, *Paroctopus*, confined to the hepatopancreas. Vitamin A was not investigated.

Recently Fisher, Kon, and Thompson (1956) have reported finding vitamin A in the livers of a European squid, *Loligo forbesi*, in higher concentrations and amounts than in the eyes. In both organs the vitamin A appears to be all-*trans* by both chemical and biological test.

[3] Neilands (1947) has reported finding vitamin A in the hepatopancreas of the American lobster. He gives the following measurements, made at the end of a nutri-

It should be noted also that no one has yet demonstrated symptoms of vitamin A deficiency in an invertebrate. Attempts to produce this condition in the cockroach and clothes moth failed (Crowell and McCay, 1937; McCay, 1938; Bowers and McCay, 1940); indeed a large body of work on insect nutrition has as yet failed to reveal any need of the fat-soluble vitamins A, D, or E (cf. Trager, 1953). "The preeminently *visual* role of vitamin A appears with great force in these animals; and indeed no evidence exists that in invertebrates it exercises other functions" (Wald, 1943).

In the vertebrate eye, vision involves a cycle of geometrical isomerization of vitamin A. The rod pigment, rhodopsin, is synthesized, over the intermediate aldehyde retinene, from a specific *cis* isomer of vitamin A called neo-*b*, and bleaches to yield all-*trans* vitamin A. This in turn—or the corresponding retinene—must be reisomerized to neo-*b* for vision to continue (Hubbard and Wald, 1952–53; Hubbard, 1955–56). The same isomerization cycle is associated with the cone pigment, iodopsin (Wald, Brown, and Smith, 1954–55).

Fisher *et al.* (1952) have made the surprising discovery that euphausiid vitamin A is only about half as potent as an equal amount of all-*trans* fish liver vitamin A, when fed to rats. It was difficult for a time to imagine what this could mean; but we know now that the various geometrical isomers of vitamin A have very different biological potencies (Ames, Swanson, and Harris, 1955). If the all-*trans* isomer is assigned the potency 100, neo-*a* (13-*cis*) has the potency 75.3, and the remaining natural isomers potencies of 22 to 24 (iso-*a* (9-*cis*); iso-*b* (9, 13-*dicis*); neo-*b* (11-*cis*)). It seemed possible therefore that crustacean vitamin A includes large proportions of low potency *cis* isomers.

We have begun by examining the vitamin A of the lobster, *Homarus americanus*. The vitamin A of the eye—and hence of the whole animal—is primarily the neo-*b* isomer. Indeed the properties of our preparations do not depart sufficiently from those of pure neo-*b* vitamin A to suggest that any other isomer is present.

This is an astonishing condition. Neo-*b* vitamin A is a sterically hindered *cis* form, the first to be found in nature (Wald *et al.*, 1955; Oroshnik, 1956; Oroshnik *et al.*, 1956). In true thermodynamic equilibrium with other isomers, it occurs in very small proportion (Hubbard, 1955–56). It is an intrinsically improbable structure, probably always requiring special energy for its formation, and special conditions for its retention. We have been unable so far to find it in any vertebrate tissue outside the eye; and in eye tissues it has never exceeded 25 to 40 per cent of the vitamin A present (P. H. Brown, N. I. Krinsky, unpublished observations). We do not yet understand how vertebrate tissues accumu-

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tional experiment lasting 48 days: vitamin A per gram, fresh weight: *Hepatopancreas*, (1) natural feeding, 83.9 ± 11.5 i.u.; (2) diet without carotene: 35.9 ± 11.0; (3) diet with added carotene: 53.3 ± 15.4. *Eyes*, (1) diet without carotene, 100.0 ± 5.6 i.u.; (2) diet with added carotene, 183.3 ± 58.1.

late and retain such high proportions of the neo-*b* isomer. That the lobster eye contains this isomer virtually alone is strange indeed. It shows that mechanisms exist which can circumvent all the apparent improbabilities of this situation, and can provide whatever neo-*b* vitamin A the tissues may require.

In this investigation we have encountered for the first time the problem of identifying and measuring the neo-*b* isomer in tissue extracts. For this reason we describe our procedures in detail.

EXPERIMENTS

Material.—All the experiments were performed in December and January, on Nova Scotia lobsters which had been kept in running sea water for 1 to 3 weeks. The eye stalks, on removal from the animals, were immediately frozen, and kept dark and frozen for 1 to 2 weeks before being used.

At the end of the eye stalk is the deep purple, almost black area containing the ommatidia, covered by the relatively soft corneal tissue (Fig. 1). This area was cut away from the remainder of the eye stalk with a razor blade. The cut was made far enough back on the stalk to include all the deeply pigmented tissue. This was collected from about 100 eyes at a time, on ice. The tissue was ground with an equal volume of anhydrous sodium sulfate until dry, then extracted repeatedly with petroleum ether until the extracts were colorless. Finally the residual tissue was extracted with acetone, which should have removed any adsorbed or protein-bound vitamin A. No vitamin A was ever detected in such acetone extracts; apparently all the vitamin A of the eye is free and extractable with petroleum ether.

The optic nerves were pulled out of the stumps of the eye stalks. This tissue was much larger in bulk than the retinas. It was treated in exactly the same way.

Hepatopancreases—four of wet weight 1.77 gm.—were ground with anhydrous sodium sulfate until dry and extracted similarly.[4]

Total Retinal Vitamin A and Carotenoids.—The retinal extract contains vitamin A, a large quantity of astaxanthin, and a small amount of carotene (apparently mainly β-).

Astaxanthin was determined by measuring the absorption spectrum of the total extract in pyridine. Almost the whole absorption in the visible region is caused by this pigment; only a small correction, of the order of 5 per cent, is required for the carotene present.

Carotene is measured after removing the astaxanthin by saponification. An aliquot of the total extract of retinas is brought into 6 per cent KOH in methanol, and incubated at 40°C. for 20 minutes. This process converts the astaxanthin to astacene. The saponification mixture is diluted with 40 per cent its volume of water, and extracted repeatedly with petroleum ether. The combined petroleum ether extracts are washed twice with 4 per cent KOH in 60 per cent methanol, to remove the last traces of astacene. They contain a yellow pigment, almost entirely epiphasic in partition with 90 per cent methanol, and possessing absorption bands in hexane at about

[4] The experiments on the hepatopancreas and the optic nerve were performed by Marilyn Thompson, whom we should like to thank for permission to cite her findings.

447 and 475 mμ (β-carotene in hexane: 450, 477 mμ). The concentration is estimated from the extinction at 450 mμ, on the basis that at this wave length β-carotene has *E* (1 per cent, 1 cm.) 2500.

Vitamin A is determined by the antimony chloride test in the non-saponifiable fraction. Too little carotene is present in this fraction to interfere with the measurement. The solution is transferred to dry chloroform, and 1 ml. mixed with 2.2 ml. of saturated antimony trichloride solution in chloroform. The spectrum is recorded immediately. A single sharp absorption band is obtained, with λ_{max} 618 mμ, characteristic of vitamin A. From its extinction the concentration of vitamin A is estimated, on the basis that *E* (1 per cent, 1 cm.) at 618 mμ is 4400 (Cawley *et al.*, 1948).

The results of such measurements, carried out with a batch of 155 eyes, are as follows: Astaxanthin, 3.0 μg. per eye; carotene, 0.144 μg. per eye; vitamin A, 1.18 μg. per eye.[5]

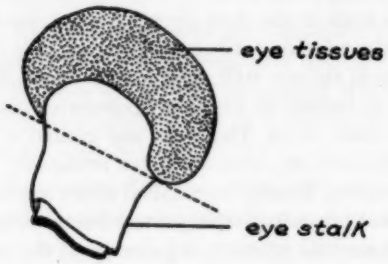

FIG. 1. Diagram of the lobster eye. The broken line shows roughly where the eye was divided so as to separate the retinal tissues, which are deeply pigmented, from the eye stalk containing the optic nerve.

Other Tissues.—No vitamin A could be detected in the optic nerve or in the combined petroleum ether and acetone extracts of the hepatopancreas.

Alcohol and Ester Fractions of Vitamin A.—The vitamin A extracted from the retina was separated into free alcohol and ester fractions by the chromatographic procedure of Ganguly *et al.* (1952). A 3 ml. aliquot of the total retinal extract in petroleum ether was adsorbed on a column of alumina (Merck, "suitable for chromatographic adsorption") which had been weakened by stirring with 5 per cent its weight of water. Then 25 ml. of 4 per cent acetone in petroleum ether were run through to elute vitamin A esters; followed by 25 ml. of 40 per cent acetone in petroleum ether to elute the free vitamin A alcohol. Each of these eluates includes portions of astaxanthin, adsorbed originally at the top of the column. To remove this, both fractions are saponified as described above, and then brought into solution in hexane. The absorption spectrum of each is measured; then each is isomerized by adding 5 μg. iodine in petroleum ether, and irradiating until no further changes occur (12 to 14 minutes)

[5] Fisher *et al.* (1954) report the following average measurements on extracts of eyes of the European lobster, *H. vulgaris*: vitamin A, 0.97 μg. per eye; carotenoids (predominantly astaxanthin with a little xanthophyll), 15 μg. per eye.

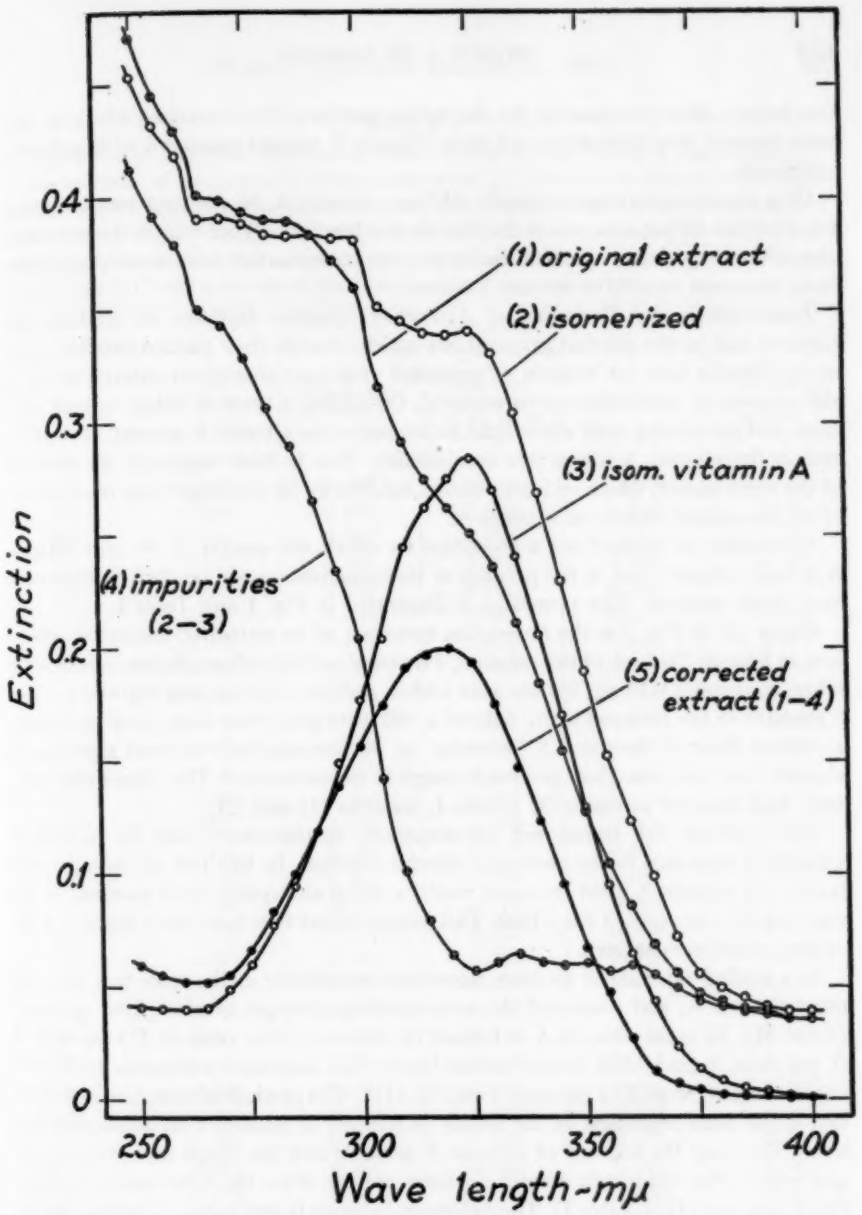

FIG. 2. Absorption spectra of lobster vitamin A and of the product of its isomerization. (1) Spectrum of an untreated extract of lobster eyes in hexane. (2) Spectrum remeasured after complete isomerization of the vitamin A with light in the presence of a trace of iodine. The concentration of vitamin A in this solution having been determined by the antimony chloride test, (3) shows the absorption spectrum of this concentration of pure, completely isomerized vitamin A. (4) The difference between this and (2), representing the spectrum of the impurities present. (5) The difference between (1) and (4), hence the absorption spectrum of the vitamin A present initially. This has the properties of neo-*b* vitamin A.

(see below). After remeasuring the absorption spectra of the isomerized solutions, they were brought into chloroform and their vitamin A content measured with antimony trichloride.

Of a sample containing originally 22.0 μg. vitamin A, 86 per cent was accounted for, of which 30 per cent was in the free alcohol fraction, 70 per cent in the ester fraction. Other experiments yielded similar results. Isomerization tests showed both fractions to consist equally of the neo-*b* isomer.

Isomerization and Derivation of Absorption Spectra.—Initially we worked with extracts and partly purified preparations which, though they yielded excellent antimony chloride tests for vitamin A, possessed very poor absorption spectra, owing to the presence of much extraneous material. On adding a trace of iodine to such solutions and irradiating with white light to isomerize the vitamin A present, the extinction in the vitamin A region rose considerably. This in itself suggested the presence of the neo-*b* isomer, which on isomerization exhibits by far the largest rise in extinction of all the natural isomers of vitamin A.

Eventually we worked out a procedure by which the spectra of the pure vitamin A initially present and of the product of its isomerization can be derived from even very crude extracts. This procedure is illustrated in Fig. 1 and Table I.

Curve (1) in Fig. 2 is the absorption spectrum of an untreated extract of lobster eyes in hexane. To 3 ml. of this solution, 2 drops of a stock iodine solution in petroleum ether, containing 0.15 μg. iodine, were added, and the solution was exposed for 2 to 3 minutes to the focussed white light of a 160 watt projection lamp passing through a neutral filter of density 1.3 (intensity *ca.* 30 foot-candles). Control experiments showed that this procedure produces complete isomerization.[6] The absorption spectrum had changed to curve (2) (Table I, columns (1) and (2)).

This solution was transferred quantitatively to chloroform and its content of vitamin A measured in the antimony chloride reaction. In this test all the geometric isomers of vitamin A yield the same result, a sharp absorption band maximal at 618 mμ with *E* (1 per cent, 1 cm.) 4400. This measurement tells how much vitamin A the original solution contained.

In a control experiment we have isomerized completely in the same way pure all-*trans* vitamin A, and measured the accompanying changes in absorption spectrum (Table II). All-*trans* vitamin A in hexane has its absorption peak at 325 mμ with *E* (1 per cent, 1 cm.) 1820. Isomerization lowers this maximum extinction to 0.94 its original height, or an *E* (1 per cent, 1 cm.) of 1711. The product of complete isomerization is the same regardless of the isomer or mixture of isomers used as starting material. Knowing the amount of vitamin A present, and the shape and height of its spectrum in the completely isomerized form, we can draw the latter as curve (3) in Fig. 2 (column (3) in Table I). The difference between it and curve (2) represents the absorption spectrum of the impurities present in the original solution (curve (4);

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[6] No directions for complete isomerization are reliable in every instance. The presence of carotenoids and other substances which absorb iodine, including at times traces of aromatic hydrocarbons in the solvent itself, may slow the reaction. The only safe procedure is to follow the isomerization spectrophotometrically until no further changes occur.

TABLE I

Derivation of Absorption Spectrum of Pure Vitamin A from That of a Crude Extract

The spectrum of the extract in hexane is measured (1); then the vitamin A in the extract is isomerized (2). An antimony chloride test shows the extract to contain 1.62 μg. per ml. vitamin A. From Table II the spectrum of this concentration of pure isomerized vitamin A is obtained (3). Columns (2) minus (3) yield the spectrum of the impurities present (4). This subtracted from (1) is the spectrum of the vitamin A present initially (5).

Wave length	Extinctions				
	(1) Extract	(2) Isomerized extract	(3) Isomerized vitamin A (1.62 μg. per ml.)	(4) Impurities	(5) Vitamin A present initially
mμ					
400	0.036	0.038	0.001	0.037	−0.001
395	0.036	0.038	0.002	0.036	0.000
390	0.037	0.041	0.003	0.038	−0.001
385	0.039	0.045	0.005	0.040	−0.001
380	0.043	0.049	0.008	0.041	0.002
375	0.047	0.056	0.013	0.043	0.004
370	0.054	0.072	0.023	0.049	0.002
365	0.067	0.098	0.043	0.055	0.012
360	0.086	0.123	0.064	0.059	0.027
355	0.104	0.152	0.095	0.057	0.047
350	0.127	0.189	0.129	0.060	0.067
345	0.154	0.229	0.170	0.059	0.095
340	0.184	0.262	0.206	0.061	0.123
335	0.211	0.304	0.240	0.064	0.147
330	0.236	0.331	0.270	0.061	0.125
325	0.250	0.341	0.284	0.057	0.193
320	0.262	0.338	0.275	0.063	0.199
315	0.275	0.340	0.262	0.078	0.197
310	0.292	0.345	0.244	0.101	0.191
305	0.316	0.351	0.209	0.142	0.174
300	0.360	0.381	0.177	0.204	0.156
295	0.372	0.382	0.148	0.234	0.138
290	0.383	0.384	0.119	0.265	0.118
285	0.389	0.383	0.094	0.289	0.100
280	0.390	0.384	0.072	0.302	0.083
275	0.394	0.387	0.059	0.328	0.066
270	0.398	0.391	0.048	0.343	0.055
265	0.402	0.391	0.041	0.350	0.052
260	0.430	0.418	0.040	0.378	0.052
255	0.047	0.432	0.040	0.392	0.055
250	0.471	0.453	0.041	0.412	0.059

also column (4) of Table I). The latter subtracted from curve (1) yields the absorption spectrum of the pure vitamin A initially present (curve (5); also column (5) in Table I).

This procedure has yielded very good results, when applied to lobster preparations

as extracted and in various stages of purification. It has of course much broader application, since it can be used generally to derive pure vitamin A spectra from solutions however contaminated. It involves the assumption that on adding a trace of iodine and irradiating, the only spectral changes observed are caused by the isomeriza-

TABLE II

Isomerization of All-Trans Vitamin A

To a solution of about 10 μg. vitamin A in 3 ml. hexane, 2 drops of hexane solution containing 0.20 μg. iodine are added. This mixture is exposed for 2 minutes to white light of intensity about 30 foot-candles.

Wave length	*E* (1 per cent, 1 cm.) of vitamin A	
	All-*trans*	Isomerized
mμ		
400	0	0
395	5	4
390	8	7
385	19	18
380	34	31
375	66	63
370	137	134
365	261	256
360	420	412
355	585	575
350	793	781
345	1055	1034
340	1310	1273
335	1530	1472
330	1715	1652
325	1820	1711
320	1743	1641
315	1664	1578
310	1564	1460
305	1361	1250
300	1133	1073
295	952	903
290	765	737
285	623	610
280	507	508
275	446	443
270	414	417

tion of vitamin A. This is apparently not a difficult criterion to satisfy. Even though carotenoids were present in some of the extracts with which we worked, this procedure yielded accurate results, probably because the carotenoids were already in the all-*trans* condition and were changed only slightly by isomerization. In general, it is safer to exclude carotenoids from the preparations.

Curve (5) in Fig. 2 presents a reasonably accurate absorption spectrum of neo-b

vitamin A. A considerable number of parameters so identify it. For comparison, the absorption spectra of pure neo-*b* vitamin A and of the product of its isomerization are shown in Fig. 3. The absorption maximum of lobster vitamin A (Fig. 2, curve (5)) lies at 319 to 320 mμ, about 5 to 6 mμ toward shorter wave lengths from the all-*trans* position. Knowing the concentration of vitamin A which yielded this spectrum, we can calculate the E (1 per cent, 1 cm.). This, in three experiments, averages 1250; in pure neo-*b* vitamin A it averages 1200. On isomerization, λ_{max} shifts from 320 to

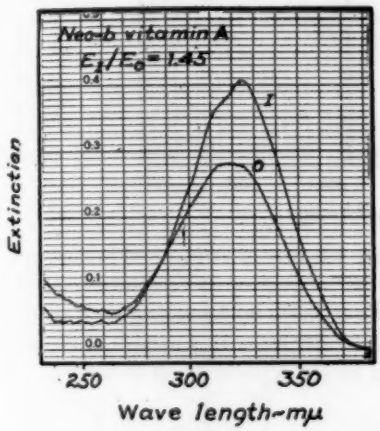

FIG. 3

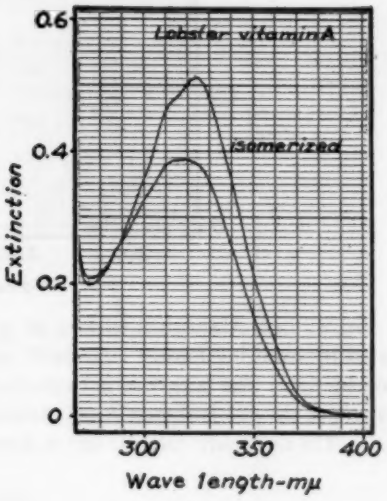

FIG. 4

FIG. 3. Absorption spectra of neo-*b* vitamin A in hexane (0) and of the product of its complete isomerization by light in the presence of a trace of iodine (I). In this figure the extinctions are for an arbitrary concentration; E (1 per cent, 1 cm.) = 1200, and is raised 1.45 ± 0.05 by isomerization, with characteristic changes in the shape of the absorption band. An isosbestic or common point appears at 287 mμ. All these changes are exhibited also by the derived spectra of lobster vitamin A (Fig. 2, curves 3 and 5), and by the directly measured spectra of a purified preparation (Fig. 4).

FIG. 4. Absorption spectra of a partly purified preparation of vitamin A from the lobster eye, dissolved in hexane, and of the product of its complete isomerization. The properties of the original preparation and its changes on isomerization are characteristic of neo-*b* vitamin A.

325 mμ, and the absorption band changes characteristically in shape, its E_{max} rising by a factor of 1.48 (average). The corresponding factor for pure neo-*b* vitamin A is 1.45 ± 0.05. As shown in Fig. 2, though the absorption rises on isomerization over most of the spectrum, it falls in the ultraviolet. A crossing point occurs at which the extinction does not change (common or isosbestic point). In the present experiments this lies at about 287 mμ, just as with pure neo-*b* vitamin A. These relationships are summarized in Table III. They show that the vitamin A of the lobster eye possesses very nearly the properties of the pure neo-*b* isomer.

Purification of Vitamin A.—An extract of lobster eyes was saponified by incubating in 6 per cent KOH at 40°C. for 20 minutes, converting all the astaxanthin present to astacene. The saponification mixture was diluted with 40 per cent its volume of water and extracted repeatedly with petroleum ether. The astacene remains behind in the

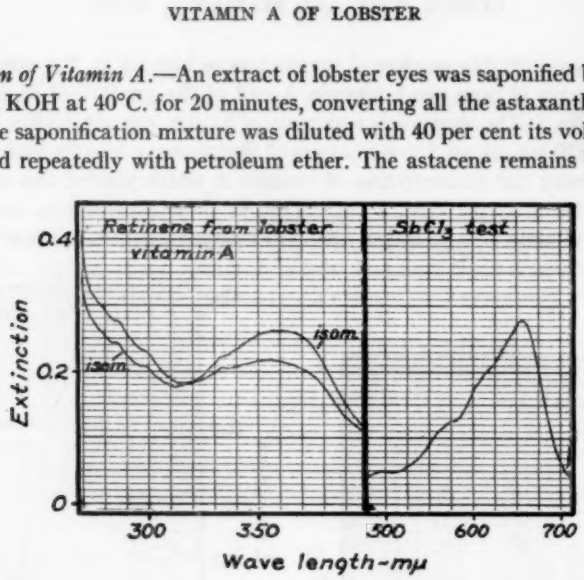

FIG. 5. *Left*, Absorption spectra of an impure solution of retinene in hexane, prepared by oxidizing lobster vitamin A, and of the product of its complete isomerization by light. The large rise in extinction of the retinene on isomerization is characteristic of the neo-*b* isomer. *Right*, Antimony chloride test with a sample of this material. The antimony chloride test is dominated by the retinene band at 666 mμ.

TABLE III

Properties of Vitamin A Extracted from the Lobster Eye, and of the Pure Neo-b Isomer

Experiments 2, 3, and 4 involve computations of the absorption properties of lobster vitamin A as described in the text. The next line shows the properties of a purified preparation of lobster vitamin A, measured directly. The last line shows the properties of pure neo-*b* vitamin A.

Material	λ_{max}	Rise of E_{max} on isomerization	E (1 per cent, 1 cm.) in hexane	Isosbestic point in isomerization
	mμ			mμ
Experiment 2	318	1.44	1290	287
Experiment 3	319	1.49	1230	286
Experiment 4	319	1.50	1220	—
Purified sample	319	1.38	1300	285
Neo-*b* vitamin A	318	1.45 ± 0.05	1200	287

saponification mixture. The combined petroleum ether layers were washed twice with fresh 6 per cent KOH in methanol diluted with 40 per cent its volume of water, to remove the last traces of astacene. The petroleum ether solution was evaporated to dryness under reduced pressure, and taken up in fresh solvent. It contained vitamin A and a small amount of carotene. It was chromatographed on a column of calcium carbonate, developed with further petroleum ether. The spectrum of each 2 ml. frac-

tion was measured as it emerged from the column. A fairly pure sample of vitamin A alcohol was collected just in front of the main carotene band. Its absorption spectrum is shown in Fig. 4, together with the spectrum following isomerization. It possessed the following properties: λ_{max} 319 to 320 mμ; E (1 per cent, 1 cm.) 1300; rise of E_{max} on isomerization, 1.38; isosbestic point on isomerization at 285 mμ. These properties are close to those of pure neo-*b* vitamin A (Table III), though departing sufficiently to suggest that slight isomerization may have occurred, perhaps caused by the saponification procedure.

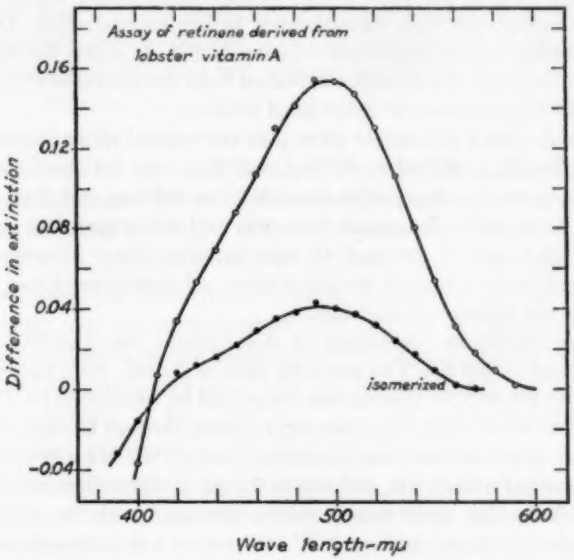

FIG. 6. Difference spectra of photosensitive pigments synthesized from retinene derived from lobster vitamin A. Of two identical samples of the retinene in digitonin solution, one was isomerized by exposure to light. Then both were mixed with excess cattle opsin, and incubated in the dark. At the end of incubation hydroxylamine was added to both solutions, and the difference spectra were measured—the difference in spectrum before and after bleaching. The unisomerized retinene had formed rhodopsin (λ_{max} 498 mμ). The isomerized sample had formed only about one-fourth as much photosensitive pigment, a mixture of rhodopsin and isorhodopsin (λ_{max} 487 mμ).

Oxidation to Retinene.—A microprocedure for this was worked out in preliminary experiments. To a sample of the purified lobster vitamin A containing 20 μg. of the vitamin in 2.5 ml. petroleum ether, 1 mg. of powdered manganese dioxide was added. This mixture was left in the dark at room temperature for 4 hours. It was centrifuged, and the clear supernatant poured off. The absorption spectrum is shown in Fig. 5. In addition to the band of retinene, it displays considerable extraneous absorption.

This preparation was isomerized with light in the presence of iodine. The absorption rose considerably, and λ_{max} shifted toward shorter wave lengths. Then 2 ml. of

this solution were transferred to 1 ml. chloroform, and 2.2 ml. antimony chloride reagent were added. The absorption spectrum of the resulting blue product is shown in Fig. 5 at the right. It displays the typical retinene band at 666 mμ, and a small inflection at about 620 mμ due to a small residue of vitamin A.

A calculation comparable with that illustrated in Fig. 2 can be performed with the data of Fig. 5. From the antimony chloride test at the right, on the basis that E (1 per cent, 1 cm., 666 mμ) = 3740, one can calculate that the retinene concentration at the left was 1.19 μg. per ml. Knowing the absorption spectrum of pure isomerized retinene, and that its E (1 per cent, 1 cm.) = 1570 (Brown and Wald, 1956), one can draw the absorption spectrum corresponding to this concentration. This, subtracted from the spectrum of the isomerized solution in Fig. 5, yields the spectrum of the impurities present; and this in turn, subtracted from the spectrum of the unisomerized solution, yields the spectrum of the original retinene.

The results of such a calculation show that the original retinene had λ_{max} 365 mμ. On isomerization, λ_{max} shifted to 367 mμ, and E_{max} rose 1.4 times. With pure neo-*b* retinene on isomerization λ_{max} shifts from 362.5 to 367 mμ, and E_{max} rises 1.7 times (Brown and Wald, 1956). It appears from this that our preparation contained about 69 per cent neo-*b* and 31 per cent all-*trans* retinene. Some isomerization had evidently occurred in the course of our procedures; yet clearly neo-*b* was still the major component of this retinene preparation.

Synthesis of Rhodopsin.—A sample of this retinene was dissolved in 2 per cent digitonin solution at pH 6.4. Two portions, each of 0.2 ml., were pipetted into microcells of capacity 0.6 ml. One portion was isomerized by irradiating for 3 minutes in the focussed light of a 160 watt projection lamp passing through Corning filters 3389 and 3962 to remove ultraviolet and heat radiation. Then 0.2 ml. of cattle opsin in 2 per cent digitonin was added to each cell, and also to 0.2 ml. of digitonin solution in a third cell to serve as blank. The opsin was in excess compared with the retinene. After 60 minutes' incubation in the dark at 23°C., 0.1 ml. of 1 M hydroxylamine was added to each cell, and the absorption spectrum was measured before and after bleaching. The difference spectra which resulted—the differences in absorption before and after bleaching—are shown in Fig. 6.

The retinene prepared from lobster vitamin A had formed rhodopsin, with λ_{max} 498 mμ. After isomerization, this retinene formed only about one fourth as much photosensitive pigment, with λ_{max} displaced to about 495 μ. Isomerization had converted the predominantly neo-*b* isomer to a mixture of isomers, predominantly all-*trans*, and containing about 25 per cent neo-*b* and iso-*a*, which formed a corresponding mixture of rhodopsin and isorhodopsin (λ_{max} 487 mμ). This is the usual result of isomerizing retinene in aqueous solution (Hubbard and Wald, 1952–53).

A calculation based on the data of Fig. 4 shows that before isomerization the retinene employed in this synthesis had E_{max} 0.121. It formed rhodopsin with E_{max} 0.152, a rise of 1.25 times. When pure neo-*b* retinene forms rhodopsin, E_{max} rises 1.5 to 1.7 times (Brown and Wald, 1956). Therefore the retinene in this preparation was 55 to 73 per cent neo-*b*. This is close to the neo-*b* content estimated from the isomerization experiment.

We can conclude that though the retinene prepared from lobster vitamin A consisted mainly of the neo-*b* isomer, some isomerization to all-*trans* had occurred in the course of our procedures. Control experiments with pure neo-*b* vitamin A show that these operations can be performed with much less isomerization; and we have no doubt that further work would show this to be true also of lobster preparations.

CONCLUSION

The vitamin A of the lobster eye has properties so close to those of pure neo-*b* vitamin A that one cannot be sure that any other isomer is present. It has already been remarked that this is a strange condition. Neo-*b* is a hindered *cis* form, normally expected to occur in very small amount. A careful examination in our laboratory of fish and cattle liver oils and cattle blood plasma has failed so far to reveal any of this isomer (P. H. Brown, unpublished observations). Direct measurement of the equilibrium established in aqueous solution in the dark between all-*trans* and neo-*b* retinene shows these isomers to be present in the proportion 95:5 (Hubbard, 1955–56). Clearly the lobster succeeds somehow in evading all the conditions that ordinarily make the neo-*b* isomer difficult to obtain and keep, and manages to store it in the eye virtually pure. One of the most puzzling problems in the chemistry of vertebrate vision is to understand how the eye obtains sufficient neo-*b* vitamin A to maintain rod and cone vision. Perhaps this will cease to be a problem, once we understand how the lobster performs the much greater feat of storing this isomer virtually alone.

Recently Dr. Kon sent us an oil extracted with petroleum ether from the eyes of the euphausiid crustacean, *Meganyctiphanes norvegica*. This was examined using the procedures described in the present paper (*cf.* also Brown and Wald, 1956). At least 90 per cent of the vitamin A was found to be in the form of the neo-*b* isomer. As in the present experiments, one could not be certain that any other isomer of vitamin A was present (Wald and Brown, 1956–57).[7]

It is possible therefore that Eucarid crustacea in general—decapods and euphausiids—store this isomer in the eye. This would explain in large part the observation of Fisher *et al.* (1952) that the vitamin A from euphausiid eyes has only about half the biological potency in rats expected of it on the basis of chemical and physical tests. Compared with all-*trans* vitamin A, the neo-*b* isomer has a biopotency of about 23 per cent (Ames *et al.*, 1955). When allowance is made for variations in bioassays performed in different laboratories, and for the possibility of small and varying amounts of other isomers of vitamin A in euphausiid oils, this discrepancy is perhaps not serious.

The hypothesis that crustacean vitamin A may in general be primarily the

[7] Kampa (1955) has reported the extraction from the eyes of *Euphausia pacifica* of a light-sensitive pigment, "euphausiopsin," with λ_{max} (difference spectrum) about 462 mμ. Following bleaching, these extracts contained vitamin A, but no retinene. Control extracts made prior to bleaching are not mentioned.

neo-*b* isomer accounts for the observation of Lambertsen and Braekkan (1955) that vitamin A extracted from the eyes of the deep sea prawn, *Pandalus borealis*, possesses an ultraviolet absorption band lying "to the left from the expected vitamin A_1 curve, $E_{310m\mu}/E_{325m\mu} = 0.926 \ldots$." In pure lobster vitamin A this ratio of extinctions in hexane is 0.96; in pure neo-*b* vitamin A, 0.97; in all-*trans* vitamin A, 0.86. Lambertsen and Braekkan also reacted their preparation with maleic anhydride, according to the procedure of Robeson and Baxter (1947). They recovered 70.6 per cent of the vitamin A. This is close to the result obtained by Robeson *et al.* (1955) with neo-*b* vitamin A (60 to 65 per cent recovery; neo-*a* 83 per cent; all-*trans*, 3 per cent). Lane (1950) has reported the extraction from the calanoid copepods *Temora turbinata* and *Centropages typicus* of a non-saponifiable fraction containing what appeared to be vitamin A, but with its absorption displaced toward shorter wave lengths than usual. Lane stated the absorption maximum to be 310 mμ; but inspection of his figure shows that though this is in fact the wave length at which he measured the highest extinction, the center of the absorption band as drawn is about 318 mμ, and the band as a whole resembles that of neo-*b* vitamin A.[8]

It should be noted finally that Fisher *et al.* (1952, 1953) have been unable thus far to identify vitamin A in the amphipods, isopods, or cladocerans they have examined, or in the copepod, *Calanus finmarchicus*. This may be due merely to low concentration, or to some as yet unrecognized factor that interferes with the measurement of vitamin A; yet it must be conceded to be as yet too early to assume that all crustacea possess vitamin A in any form, even in their eyes. It may be mentioned in this connection that attempts some years ago to extract vitamin A from the heads of insects—*Drosophila*, the grasshopper *Melanoplus*, the dragonfly *Sympetrum*—failed to yield a clear test for this substance though relatively large amounts of tissue (6 to 14 gm.) were employed (Wald, unpublished observations).

SUMMARY

In many crustacea, including the lobster, the bulk of the vitamin A of the whole animal is concentrated in the eyes. Recently Fisher, Kon, and Thompson found that vitamin A extracted from the eyes of euphausiid crustacea has only

[8] Fisher *et al.* (1955 *a*) have recently reported the extraction and partial purification from the eyes of the euphausiid, *Meganyctiphanes norvegica*, of what is apparently a *cis* isomer of vitamin A, yielding the typical antimony chloride test for vitamin A, but possessing initially λ_{max} in hexane at 311 to 312 mμ. On isomerization λ_{max} moves to 325 mμ, and the extinction rises about 36 per cent. This substance may account for only a few per cent of the total vitamin A of the eye (Dr. Kon, personal communication). Its properties are close to those of neo-*c* vitamin A (11, 13-*dicis*) (Wald *et al.*, 1955; Oroshnik *et al.*, 1956).

about one half the biological potency of the same amount of the all-*trans* acetate or fish liver vitamin A.

In the present experiments the vitamin A of the lobster eye is found to consist almost entirely of the hindered *cis* isomer, neo-*b*, the precursor in the vertebrate retina of the visual pigments rhodopsin and iodopsin. This isomer is known to have a low biological potency in the rat, only about one quarter that of all-*trans* vitamin A. In the lobster eye it is virtually all extractable with petroleum ether, about 30 per cent in the form of free alcohol, about 70 per cent in the form of esters. It was identified by its absorption spectrum, as derived from measurements on crude extracts, and measured directly in purified preparations; the changes in absorption which accompany isomerization; oxidation to the corresponding retinene; and synthesis from the latter of rhodopsin.

The examination of an extract of euphausiid eyes, provided by Dr. Kon, also revealed the presence of neo-*b* vitamin A virtually alone. This may be the characteristic condition in the eyes of Eucarid crustacea. It is peculiar in that the neo-*b* isomer, being a sterically hindered form, is ordinarily expected to be represented in any equilibrium mixture of geometric isomers in very small amount. Apparently certain crustacea have ways of circumventing the difficulties implicit in producing and retaining this isomer, and store it in the eye virtually alone.

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THE VITAMIN A OF A EUPHAUSIID CRUSTACEAN

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(Received for publication, November 12, 1956)

Among crustacea, the euphausiids appear to be particularly rich in vitamin A, almost all of which is concentrated in the eyes. In some species no vitamin A at all has been detected in other tissues, or in whole bodies less the eyes. The vitamin A is peculiar in possessing a very much lower nutritional potency in rats than is exhibited by all-*trans* or fish liver vitamin A (Batham *et al.*, 1950; Fisher, Kon, and Thompson, 1952, 1954, 1955).

The low biopotency of euphausiid vitamin A implies that it may consist largely of low potency *cis* isomers (*cf.* Kon's suggestion, 1954). The neo-*a* isomer (13-*cis*) has about 73 per cent the potency of all-*trans* vitamin A, the remaining natural *cis* isomers only 22 to 24 per cent (neo-*b*, iso-*a*, iso-*b*) (Ames *et al.*, 1955).

Recently it has been shown that lobster vitamin A, almost all of which is in the eyes, consists almost entirely of the neo-*b* isomer (11-*cis*) (Wald and Burg, 1955, 1956–57).

Dr. Kon has sent us for examination an oil extracted from the eyes of the euphausiid, *Meganyctiphanes norvegica*. 24 gm. of eyes, representing several thousand animals, were extracted with petroleum ether containing 23 per cent alcohol in a Waring blendor (*cf.* Fisher *et al.*, 1952). The solvent was evaporated off, and the oil sealed under nitrogen. It was in this form that we received it. It was examined by the same procedures as used with the lobster material (Wald and Burg, 1956–57).

A sample of the oil was saponified at room temperature (about 26°C.) for 2¾ hours in 6 per cent KOH in methanol. This mixture was diluted with 40 per cent its volume of water, and extracted repeatedly with petroleum ether. The absorption spectrum of the petroleum ether solution is shown in Fig. 1. It displayed a broad absorption maximum at about 317 mμ, which moved to 324 mμ on isomerization with light in the presence of a trace of iodine. Simultaneously E_{max} rose by a factor of 1.39, and the absorption band changed characteristically in shape. These observations with a relatively crude preparation already indicate that the bulk of this material is neo-*b* vitamin A (Brown and Wald, 1956).[1]

* This investigation was supported in part by funds from The Rockefeller Foundation and the Office of Naval Research.

[1] These isomerizations are slow compared with those reported previously (Brown and Wald, 1956; Wald and Burg, 1956–57). The reason is that while the earlier isom-

This preparation displayed absorption not due to vitamin A, the magnitude of which can be estimated by a procedure described earlier (Wald and Burg, 1956–57). This involves determining by means of an antimony chloride test the vitamin A concentration of the isomerized solution. The absorption spectrum of this concentration of isomerized vitamin A being known, the difference between it and the spectrum of the

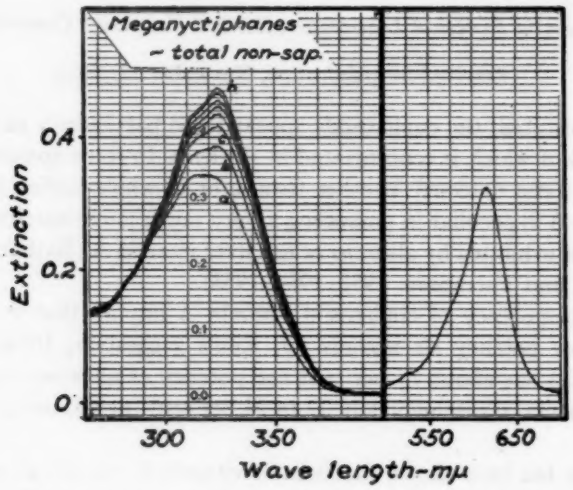

FIG. 1. The total non-saponifiable fraction of an extract of *Meganyctiphanes* eyes. *Left*, (*a*) absorption spectrum of the extract in 3 ml. petroleum ether. It displays the broad absorption band maximal at about 317 mμ characteristic of neo-*b* vitamin A. Iodine was added (2 μg. in a droplet of petroleum ether) and the solution was irradiated with white light of intensity about 30 foot-candles. Curve (*b*) was recorded after 2 minutes' irradiation, (*c*) after a total of 4, (*d*) 6, (*e*) 8, (*f*) 10, (*g*) 15, and (*h*) 25 minutes. The extinction rose, λ_{max} moving to 324 mμ, and the band became asymmetrical—all changes characteristic of the isomerization of neo-*b* vitamin A under these conditions. *Right*, 1 ml. of the isomerized solution after it had been transferred to 1 ml. chloroform was mixed with 2.2 ml. antimony chloride reagent, and the spectrum of the resulting blue product recorded at once, beginning at 700 mμ. It displays the absorption band at 618 mμ characteristic of vitamin A.

isomerized preparation represents the absorption caused by impurities. This subtracted from the spectrum of the unisomerized preparation yields the corrected spectrum of the unisomerized vitamin A.

As an example, this computation can be performed with the data of Fig. 1. 1 ml. of the isomerized preparation was transferred to 1 ml. chloroform and mixed with

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erizations had been performed in purified hexane, the present ones were done in petroleum ether contaminated with aromatic hydrocarbons in amounts sufficient to bind much of the added iodine.

2.2 ml. antimony chloride reagent. The resulting blue product had E_{max} 0.322 (618 mμ). Since the E (1 per cent, 1 cm.) of vitamin A in this test is 4400, the concentration of vitamin A was (0.322/4400) × 10,000 = 0.733 μg. per ml. The 3.2 ml. of the test mixture therefore contained 3.2 × 0.733 = 2.34 μg. vitamin A. The vitamin A content of the solution in Fig. 1 was therefore 2.34 μg. per ml. Since completely isomerized vitamin A has E (1 per cent, 1 cm.) 1711 at 325 mμ, this concentration should have E_{max} (2.34/10,000) × 1711 = 0.400. However, the E_{max} of the isomerized solution in Fig. 1 is 0.474. The difference, 0.074, is the extinction at 325 mμ caused by impurities. In the same way, knowing the extinction of pure isomerized vitamin A at 318 mμ, one finds that the extinction caused by impurities at that wave length is 0.075, and that the E_{max} of the unisomerized vitamin A is 0.342 − 0.075 = 0.267. Therefore E_{max} rises on isomerization 0.400/0.267 = 1.50 times. Also since 2.34 μg. per ml. of the unisomerized vitamin A had E_{max} 0.267, its E (1 per cent, 1 cm.) was (10,000/2.34) × 0.267 = 1140.

TABLE I

Constants Associated with the Absorption Spectrum of Meganyctiphanes Vitamin A, and Its Changes on Isomerization, Compared with Corresponding Constants of Neo-b Vitamin A

The *Meganyctiphanes* preparations include the total non-saponifiable, and two partially purified fractions from a chromatographic adsorption on alumina. λ_{max} and λ_{sub} are respectively the maxima of the main and the subsidiary absorption bands.

Preparation	λ_{max}	λ_{sub}	Rise of E_{max} on isomerization	E (1 per cent 1 cm.)	Isosbestic point
					mμ
Total non-saponifiable	317	—	1.50	1140	—
1st fraction	317	234	1.44	1190	288
2nd fraction	318	234	1.41	1220	289
Averages			**1.45**	**1183**	
Neo-*b* vitamin A	318	233	1.45 ± 0.05	1200	287

In this way one can compute the λ_{max} and E (1 per cent, 1 cm.) before isomerization, and the rise of E_{max} on isomerization. A further constant, which can be read from the uncorrected data, is the crossing point of the curves for unisomerized and isomerized vitamin A; *i.e.*, the common or isosbestic point. In all these parameters, *Meganyctiphanes* vitamin A is identical, within the limits of error, with pure neo-*b* vitamin A (Table I). A marked change in the *shape* of the absorption band on isomerization, apparent in Figs. 1 and 2, also is characteristic of the neo-*b* isomer (*cf.* Brown and Wald, 1956).

A portion of the *Meganyctiphanes* vitamin A was partly purified by chromatographic adsorption. A large sample of the oil was saponified, the non-saponifiable fraction extracted with petroleum ether, and absorbed on a column of alumina (Merck reagent, "suitable for chromatographic analysis") weakened with 5 per cent its weight of water. The chromatogram was developed with 10 per cent acetone in petroleum ether. The first two fractions to run through the column contained the bulk of the vitamin A. Absorption spectra of the

first filtrate in hexane are shown in Fig. 2. This fraction accounted for half the vitamin A in the total eluate. It possessed initially λ_{max} 317 mμ, with a subsidiary band at 234 mμ. On isomerization, λ_{max} moved to 325 mμ, and E_{max} rose 1.38 times. Also the subsidiary band disappeared, the extinction at 234

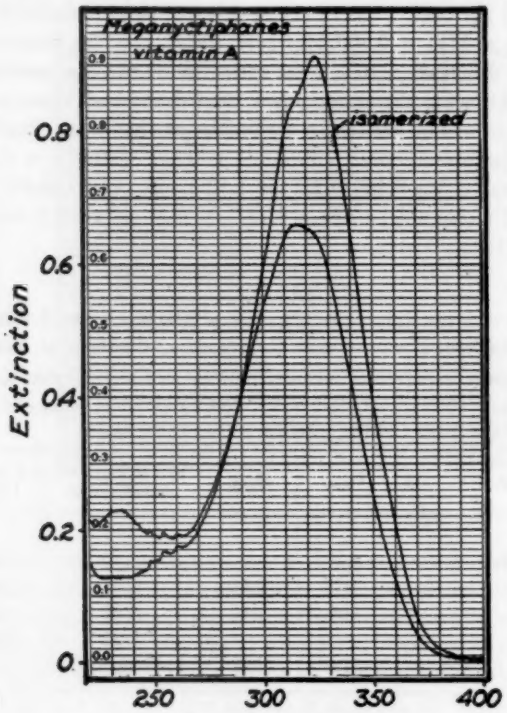

FIG. 2. Absorption spectra in hexane of an extract of *Meganyctiphanes* eyes, partly purified by saponification and chromatographic adsorption on alumina. The spectrum originally displays the low, broad absorption band maximal at 317 mμ and the subsidiary band at 234 mμ characteristic of neo-*b* vitamin A. On isomerization with light in the presence of iodine, the main band rises in height, moves to λ_{max} 324 mμ, and becomes sharper and highly asymmetrical, while the subsidiary peak at 234 mμ is eliminated—all changes characteristic of the isomerization of neo-*b* vitamin A.

mμ falling to 0.56 its former value; in pure neo-*b* vitamin A this factor is 0.55. The second filtrate fraction was less pure, but yielded essentially the same results. These two fractions accounted for 90 per cent of the total eluate, or 75 per cent of the vitamin A originally adsorbed. The data from both fractions were corrected for extraneous absorption as already described. The constants which emerged are summarized in Table I. Again, they are virtually identical with constants obtained with pure neo-*b* vitamin A.

Two samples of free *Meganyctiphanes* vitamin A alcohol, prepared by saponification, were oxidized to the corresponding retinene. The vitamin A in petroleum ether solution was let stand for 5 and 8 days in the refrigerator with 17 times its weight of manganese dioxide powder prepared according to Attenburrow *et al.* (1952). The absorption spectrum of one such product is shown in Fig. 3. It had λ_{max} in hexane at 362 mμ. On isomerization by light in the pres-

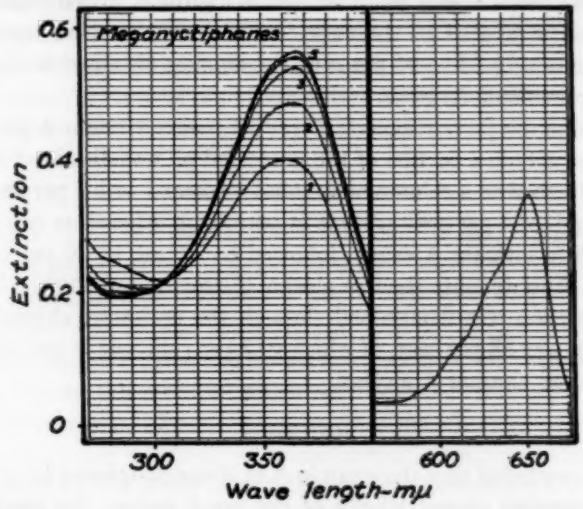

FIG. 3. Absorption spectra of retinene prepared by the oxidation of *Meganyctiphanes* vitamin A. *Left*, curve (1) is the spectrum of a petroleum ether solution which had to this point been protected from light. To 1 ml. of this solution, 0.15 μg. iodine was added in a droplet of petroleum ether, and the solution was irradiated with white light of intensity about 30 foot-candles (2) for 5 minutes, (3) for a total of 10, (4) 15, and (5) 20 minutes. The rise of extinction of the main band and displacement of λ_{max} to longer wave lengths are characteristic of the isomerization of neo-*b* retinene. *Right*, 1 ml. of the isomerized retinene was transferred to 1 ml. chloroform, 2.2 ml. of antimony chloride reagent were added, and the spectrum was recorded at once, beginning at 700 mμ. The resulting blue product had λ_{max} 656 mμ, characteristic of retinene when recorded so quickly.

ence of iodine, E_{max} rose 1.41 times, and λ_{max} shifted to 367 mμ.[1] The second preparation yielded similar results. Antimony chloride tests displayed the absorption band characteristic of retinene.[2] When the concentrations had

[2] The antimony chloride test with retinene, as usually recorded in the past in this laboratory, yields an absorption maximum at 664 to 666 mμ. In the present instance λ_{max} is about 656 mμ. The principal reason for this is more rapid recording. When such recording is started, as here, at 700 mμ, the absorption peak is reached within 7 to 8 seconds after mixing the reagents, and lies at about 656 mμ. In about the next 20 seconds the peak rises somewhat in height, and moves to 664 to 666 mμ.

been determined through the antimony chloride tests, these data could be corrected for the presence of extraneous absorption in the way already described for vitamin A, with the result that in these two preparations isomerization raised E_{max} 1.58 and 1.64 times. For pure neo-*b* retinene this factor is 1.7; so that following saponification and oxidation on manganese dioxide these preparations apparently contained about 84 and 92 per cent of the neo-*b* isomer.

One of these preparations was dissolved in digitonin, mixed with cattle opsin in excess, and incubated in the dark for 2.3 hours at room temperature. It formed rhodopsin, in 88 to 96 per cent the amount obtained in similar experiments with pure neo-*b* retinene.

To determine the proportions of *Meganyctiphanes* vitamin A present as free alcohol and as esters, a portion of the untreated oil was dissolved in petroleum ether, and adsorbed on a column of alumina weakened with 5 per cent its weight of water. 25 ml. of 4 per cent acetone in petroleum ether were run through the column to elute vitamin A esters, followed by 25 ml. of 40 per cent acetone in petroleum ether to elute the free alcohol (Ganguly *et al.*, 1952). The vitamin A in each fraction was determined through the antimony chloride test. The total recovery was 88 per cent, of which 90.7 per cent was in the ester fraction, 9.3 per cent free vitamin A alcohol.

DISCUSSION

It may be concluded that the vitamin A of *Meganyctiphanes* eyes, like that of the lobster, consists almost wholly of the neo-*b* isomer. Its properties after saponification do not depart sufficiently from those of pure neo-*b* vitamin A to indicate that any other isomer is present. The measurements by which this isomer was identified involve limits of variability such that at most 10 per cent of the vitamin A could have been in other forms. This information, which was communicated to Dr. Kon, has since been confirmed in his laboratory (Plack *et al.*, 1956).

Fisher *et al.* (1955 *b*) have described the isolation from *Meganyctiphanes* eyes of a vitamin A with λ_{max} 311 to 312 mμ in hexane. On isomerization with light in the presence of iodine, λ_{max} moved to 325 mμ, and E_{max} rose 1.36 times. These properties are close to those of neo-*c* vitamin A (11, 13-*dicis*), for which λ_{max} in hexane is 311 mμ, and E_{max} rises 1.36 times on isomerization (Wald *et al.*, 1955; Oroshnik *et al.*, 1956). This substance accounts for only a few per cent of the vitamin A of *Meganyctiphanes* eyes (Plack *et al.*, 1956). For this reason, and because of the fact that among the geometric isomers of vitamin A its properties most resemble those of neo-*b*, it can readily be accommodated within our measurements.

The only remaining discrepancy with our observations seemed for a time to involve bioassays. Fisher *et al.* (1952, 1955 *b*) had reported that vitamin A from *Meganyctiphanes* eyes possesses on the average about 60 per cent the

biological activity of all-*trans* vitamin A. Ames *et al.* (1955) assess the biopotency of neo-*b* vitamin A at about 23 per cent of all-*trans*. If, as we find, *Meganyctiphanes* vitamin A is at least 90 per cent neo-*b*, it should possess a considerably lower biopotency than had been observed.

Since this information was communicated to Dr. Kon, the bioassays have been reexamined in his laboratory, with the result that they have been very considerably revised. *Meganyctiphanes* vitamin A is now found to assay at 32 per cent of all-*trans*; while those components of it which react slowly with maleic anhydride, and which include the neo-*b* isomer, assay at 25 per cent of all-*trans* vitamin A (Plack *et al.*, 1956). Little if any discrepancy in this regard remains therefore with the present observations.

SUMMARY

The vitamin A of the euphausiid crustacean, *Meganyctiphanes norvegica*, consists almost wholly of the hindered *cis* isomer, neo-*b* (11-*cis*). In this animal vitamin A is concentrated almost entirely in the eyes; and its properties so closely resemble those of pure neo-*b* vitamin A as not in themselves to indicate that any other isomer is present. However, Fisher *et al.* (1955 *b*) have isolated a small fraction from this material which may be neo-*c* vitamin A (11, 13-*dicis*). The neo-*b* isomer was identified by its absolute absorption spectrum, the changes of absorption spectrum on isomerization, oxidation to neo-*b* retinene, and synthesis from the latter of rhodopsin. This identification is also in good accord with new, revised bioassays of *Meganyctiphanes* vitamin A by Plack *et al.* (1956).

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REVERSIBLE CHANGES OF PROTEIN CONFIGURATION IN STIMULATED NERVE STRUCTURES*

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(Received for publication, October 27, 1956)

The chemical events of excitation have received considerably less attention than their physical counterparts. This comparative neglect can probably be explained by the remarkable suitability of physical methods for the study of short lasting phenomena, contrasting with the lack of chemical methods capable of detecting rapidly reversible changes.

However, the importance of the chemical aspects of the excitation problem is more and more widely recognized, as witnessed by the increasing amount of work devoted to the ionic shift, the energy-yielding reactions, and other chemical changes associated with excitation.

The present paper deals with alterations of proteins induced by stimulation of nerves and nerve centers. The over-all problem of the chemistry of cellular excitation will be surveyed in a review to be published (Ungar, 1957).

A number of observations indicate a high rate of protein breakdown in nerve structures submitted to prolonged and intense stimulation (Soula, 1912, 1913; Soula and Faure, 1913; Hydèn, 1943, 1955; Geiger *et al.*, 1953). More subtle and possibly reversible changes in protein structure have been postulated by Meyer (1937), McElroy (1947), Nassonov (1948), and Segal (1956) without, however, the support of direct experimental evidence. It is believed that the experiments reported in this paper have produced some evidence in favor of reversible molecular rearrangements taking place in the proteins of stimulated structures.

Methods

Electrical stimulation was used in all experiments by means of a 60 cycle, 40 volt alternating current from a Harvard stimulator connected to platinum electrodes. Frog and rat sciatic nerves were excised, placed in the appropriate Ringer solution, and stimulated for the specified interval. Stimulation of the cerebral cortex in cats and dogs was produced with the technique described by Geiger, Dobkin, and Magnes (1953) through electrodes placed on the brachial plexus. The unstimulated side was

* Some of the results reported in this paper were presented at the International Physiological Congress in Brussels (Ungar *et al.*, 1956).

used as control. Rat cortex was stimulated by one electrode placed on each sciatic nerve.

Stimulated tissues, as well as unstimulated controls, were frozen with dry ice contained in a metal cylinder closed with a copper plate at one end (Geiger *et al.*, 1956). The frozen tissue was rapidly weighed on a Roller-Smith balance and ground up in a mortar with sand and saline. The tissue homogenate was kept 20 to 30 minutes in a water bath at 37.5°C., centrifuged, and its supernatant used for the protein studies.

The criterion for modification of protein structure was based on the work of Crammer and Neuberger (1943) demonstrating a shift of the ultraviolet spectrum in urea-denatured proteins. In alkaline solution (above pH 12) these proteins showed a dis-

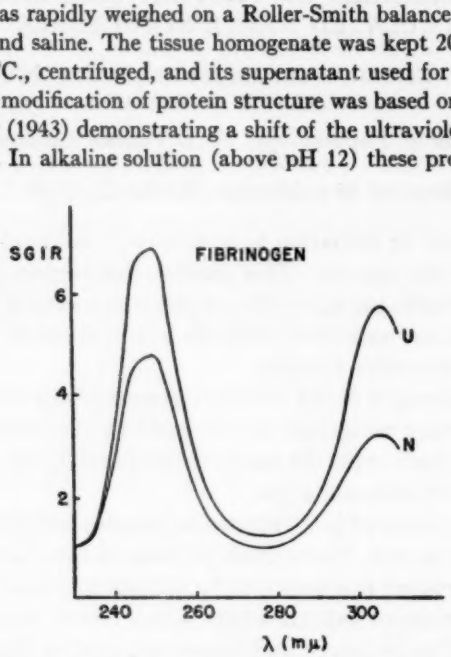

FIG. 1. Side-group ionization ratio in native and denatured fibrinogen. Bovine fibrinogen (fraction I of Armour Laboratories) dissolved in 0.85 per cent NaCl. *N*, native protein; *U*, protein denatured in 6 M urea for 2 hours at room temperature. Final concentration of solutions was 0.025 per cent.

placement of the absorption peak from 275 to 280 mμ to 295 to 300 mμ and a marked increase of the extinction coefficient. These observations have been confirmed and extended by Tanford and Roberts (1952).

In the present experiments, tissue extracts were diluted to a concentration corresponding to 2 to 5 mg. of fresh tissue per ml. and their ultraviolet absorption was measured in the Beckman model DU spectrophotometer in the range of 230 to 320 mμ, both at neutrality and at pH 12.0–12.3 (in 0.25 N KOH). It was thought that the results obtained with this method could, for the purposes of the present work, be adequately expressed by the ratio $O.D._{pH\ 12}/O.D._{pH\ 7}$. This ratio will tentatively be called "side-group ionization ratio" (SGIR), for reasons discussed below. Fig. 1 illustrates the use of SGIR; it shows the ratio plotted against wave length in a solution of native fibrinogen and fibrinogen denatured in 6 M urea. Both native and denatured proteins show a peak in the 300 to 305 mμ region and another peak at 245 to 250 mμ. The first peak corresponds, according to current opinion, to the ionization of the

phenolic hydroxyl of tyrosine. The significance of the second peak will be discussed in a later section. As seen in Table I, all proteins investigated show these two peaks of the SGIR and the peaks are increased by urea. Denatured trypsin showed increase only at 300 to 305 mμ.

Protein breakdown was estimated by measuring the ultraviolet absorption of tissue extracts at 280 mμ before and after precipitation by trichloracetic acid (at the final concentration of 5 per cent, centrifuged at least 1 hour after addition). The results are expressed in per cent TCA—soluble fraction. Total concentrations of protein are given in terms of optical density units per milligram of fresh tissue.

Protease activity of brain extracts was measured in terms of the hydrolysis of N-acetyl-L-tryptophan ethyl ester[1] by a manometric method in the presence of 0.015 M bicarbonate in atmosphere of 95 per cent N and 5 per cent CO_2 (pH = 7.4). The

TABLE I

Side-Group Ionization Ratios (SGIR) of Some Proteins

	245 mμ		250 mμ		275 mμ		300 mμ		305 mμ	
	N*	D‡	N	D	N	D	N	D	N	D
Fibrinogen	4.49	6.52	4.78	6.92	0.94	1.22	2.87	5.03	3.88	5.73
Bovine γ-globulin	6.42	6.70	7.28	7.70	1.11	1.18	4.22	5.41	6.00	10.00
Bovine fraction III	4.76	5.59	5.26	6.30	1.11	1.20	2.95	4.65	3.08	6.33
Trypsin	8.05	7.85	8.80	7.83	1.35	1.35	4.22	5.90	5.77	9.00
Horse serum albumin	6.02	7.50	6.85	8.70	1.32	1.73	4.69	6.36	4.38	6.30

* Native.

‡ Denatured in 6 M urea for 2 hours at room temperature.

substrate concentration was 0.005 M. The reaction proceeded linearly for at least 1 hour and the results are expressed in micromoles of substrate hydrolyzed per hour per gram of fresh tissue.

Whenever results are given in mean values, these are followed by their standard deviation. Significance between means was calculated by Student's *t* test and the degree of significance is expressed in terms of the probability (*P*) that the means belong to the same population.

RESULTS

1. Sciatic Nerve.—The first series of experiments was done with frog sciatic nerves. After destruction of the brain and spinal cord, both sciatics were excised, rapidly weighed, and each placed in 2 ml. of Ringer solution (for frogs). After about 15 minutes' rest, one nerve was stimulated electrically for a definite interval while the other was kept as a control. Both were frozen with dry ice before extraction; the stimulated nerve was frozen while still stimulated. Each

[1] Prepared by Dr. S. L. Shapiro of the Department of Organic Chemistry, U. S. Vitamin Corporation.

experiment represents the pooled extracts from four frogs. Fig. 2 shows the mean SGIR plotted against wave length in the control nerves, the stimulated nerves, and in a control nerve extract in which the proteins were denatured in 6 M urea. In frog sciatic, stimulation caused an increase of the SGIR at 300 mμ. At 245 mμ the stimulation had no effect although the SGIR could be raised by urea. Table II lists the ratios obtained in individual experiments with varying duration of stimulation. Even 5 seconds' stimulation increased

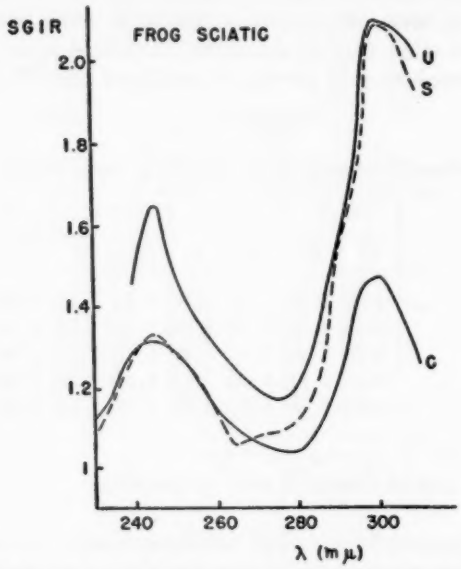

FIG. 2. Mean SGIR of frog sciatic nerve extracts from resting nerves (*C*), stimulated nerves (*S*), and after urea denaturation of a resting nerve extract (*U*) (see Table II).

the ratio; shorter stimuli did not prove practicable under the conditions of the experiment. The difference between the mean SGIR of control and stimulated nerves was found significant.

Similar experiments were performed with rat sciatic nerves taken from rats killed by decapitation. The nerves were kept in mammalian Ringer solution, two pairs of nerves were used in each experiment, and the duration of stimulus was 30 seconds in all experiments. In all other respects, the technique was the same as in the frog experiments.

Table III summarizes the results and shows that at 254 mμ the SGIR was consistently and significantly higher in stimulated nerves than in the controls. At 305 mμ the effect was more irregular. The differences would be valid even without statistical analysis since the control and stimulated nerves come from

TABLE II

Ultraviolet Absorption (300 mμ) in Resting and Stimulated Frog Sciatic Nerve Extracts

Duration of stimulus	Control			Stimulated			Δ SGIR
	O.D.		SGIR	O.D.		SGIR	
	Neutral	Alkaline		Neutral	Alkaline		
sec.							
5	0.152	0.236	1.55	0.148	0.252	1.72	0.17
10	0.168	0.253	1.53	0.170	0.282	1.67	0.14
10	0.179	0.220	1.23	0.151	0.281	1.87	0.64
30	0.162	0.241	1.50	0.134	0.322	2.40	0.90
60	0.150	0.220	1.47	0.168	0.341	2.03	0.56
120	0.149	0.218	1.46	0.152	0.413	2.71	1.25
120	0.150	0.222	1.48	0.145	0.309	2.13	0.65
Mean.....	0.159	0.230	1.45	0.153	0.314	2.05	0.60
± S.D.....			0.11			0.38	
P.....						0.01–0.001	

TABLE III

Effect of Stimulation on Rat Sciatic Nerve

No.	Side-group ionization ratio					
	254 mμ			305 mμ		
	Control	Stimulated	Δ	Control	Stimulated	Δ
1	1.41	1.89	0.48	1.61	1.49	−0.12
2	1.56	1.89	0.33	1.47	1.51	0.04
3	1.41	1.67	0.26	1.81	1.92	0.11
4	1.41	2.17	0.76	1.41	1.94	0.53
5	1.33	1.74	0.41	1.59	1.75	0.16
6	1.27	1.89	0.62	1.49	1.92	0.43
7	1.35	1.79	0.44	1.75	1.89	0.14
8	1.41	1.79	0.38	1.37	1.43	0.06
Mean.....	1.39	1.85	0.46	1.56	1.73	0.17
± S.D.....	0.08	0.16		0.16	0.22	
P.....		0.001				

Duration of stimulus 30 seconds.

the same individuals and the results can be paired. Fig. 3 shows the spectrum of SGIR in control, stimulated, and urea-denatured nerve extracts.

The results just described indicate that electrical stimulation causes some modification in nerve proteins which can be detected by an increase, at certain critical wave lengths, of the ultraviolet absorption of alkaline extracts. It remained important to ascertain whether these changes were not due, in some

way, to the direct action of the electrical current on the nerve. In some experiments, discarding portions of the nerve directly in contact with the electrodes failed to influence the results.

2. Cerebral Cortex.—The direct effect of the electric current could be eliminated by afferent stimulation of a nerve center. This was done in the sensorimotor area of one hemisphere excited through electrical stimulation of the brachial plexus; the same area of the opposite hemisphere was used as control. In cats, anesthetized with nembutal, the brain was exposed and the control

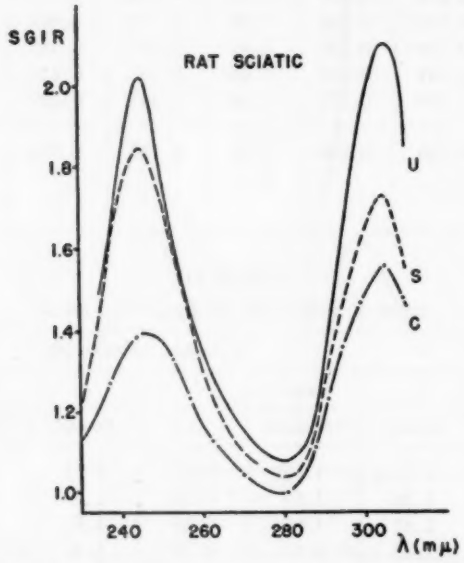

FIG. 3. Mean SGIR of rat sciatic nerve extracts from resting nerves (*C*), stimulated nerves (*S*), and after urea denaturation of a resting nerve extract (*U*) (see Table III).

cortex was frozen and excised. Immediately afterwards, the brachial plexus was stimulated for 30 seconds and the corresponding cortical area frozen and removed. The samples of cortex were then treated as described above.

Table IV shows the results of these experiments. It was not entirely unexpected that the resting SGIR should show wider variations in the cortex than in the isolated nerve. If each experiment is considered as having its own control, stimulation consistently resulted in rise of the SGIR at 245 and 300 mμ. The mean ratios were significantly different at 300 mμ but not at 245 mμ. Fig. 4 shows the SGIR-spectrum and the effect of urea on the cortical extract. It also shows the averages of four similar experiments performed in dogs; here, the effect of stimulation was less marked than in cats. Experiments involving stimulation of the brain were also done in rats, in conjunction with other studies. These experiments will be described in a later section.

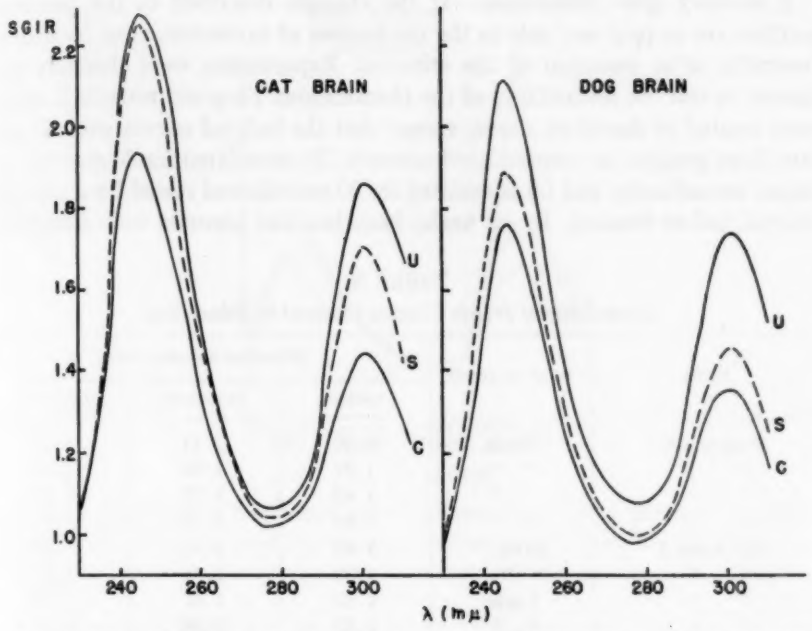

FIG. 4. Mean SGIR of extract of cerebral cortex. Resting cortex (*C*), after stimulation of the corresponding brachial plexus (*S*), urea-denatured cortical extract (*U*). Left, cat brain (see Table IV); right, dog brain.

TABLE IV

Effect of Afferent Stimulation (Brachial Plexus) on Cerebral Cortex of Cats

No.	Side-group ionization ratio					
	245 mμ			300 mμ		
	Control	Stimulated	Δ	Control	Stimulated	Δ
1	2.21	2.38	0.17	1.68	1.83	0.15
2	2.06	2.88	0.82	1.82	2.47	0.65
3	2.07	2.46	0.39	1.47	1.70	0.23
4	2.86	3.15	0.29	1.61	1.67	0.06
5	1.79	2.06	0.27	1.39	1.82	0.43
6	2.40	2.78	0.38	1.30	1.78	0.48
7	1.70	2.09	0.39	1.22	1.43	0.21
Mean.....	2.16	2.54	0.39	1.50	1.81	0.32
± S.D.....	0.39	0.41		0.22	0.22	
P.....		0.2–0.1			0.05–0.02	

Duration of stimulus 30 seconds.

3. Recovery after Stimulation.—If the changes described in the preceding sections are to play any role in the mechanism of excitation, they have to be reversible after cessation of the stimulus. Experiments were therefore performed to test the reversibility of the phenomenon. Frog and rat sciatic nerves were treated as described above, except that the isolated nerves were divided into three groups: (*a*) unstimulated controls, (*b*) stimulated for 30 seconds and frozen immediately, and (*c*) stimulated for 30 seconds and rested for a specified interval before freezing. In cat brain, both brachial plexuses were stimulated

TABLE V

Reversibility of Protein Changes Produced by Stimulation

Tissue	Recovery period	Side-group ionization ratio		
		Control	Stimulated	Recovered
Frog sciatic*	2 min.	1.40	2.11	1.42
" "	" "	1.27	1.88	1.28
" "	" "	1.43	1.77	1.27
" "	" "	1.42	1.64	1.23
Rat sciatic‡	30 sec.	1.42	1.72	1.59
" "	" "	1.30	1.76	1.65
" "	1 min.	1.37	1.81	1.30
" "	" "	1.52	1.85	1.50
" "	2 min.	1.33	1.80	1.28
" "	" "	1.53	1.88	1.47
Cat brain‡	2 min.	2.02	2.50	1.98
" "	" "	1.58	1.83	1.57
" "	" "	1.65	2.03	1.57

Duration of stimulus 30 seconds.
* SGIR at 300 mμ.
‡ SGIR at 245 mμ.

for 30 seconds; one sensorimotor area was frozen and excised immediately after stimulation while the opposite side was frozen after a rest interval of 2 minutes. An unstimulated control sample was taken from the parieto-occipital zone.

The results of these experiments are summarized in Table V. It is seen that the frog sciatic recovers completely from the effects of the stimulus within 2 minutes. The mean SGIR of four experiments is shown in Fig. 5. In rat sciatic, the rest interval varied from 30 seconds to 2 minutes. Apparently, at 30 seconds the recovery was not complete but at 1 and 2 minutes, the SGIR returned to normal. In cat brain, the reversal was also complete (Fig. 6).

4. Protein Breakdown in Stimulated Nerve.—It was shown by Hydèn (1943, 1955) that prolonged stimulation of nerve centers produced a depletion in protein and nucleoprotein. Since this phenomenon has not been described in

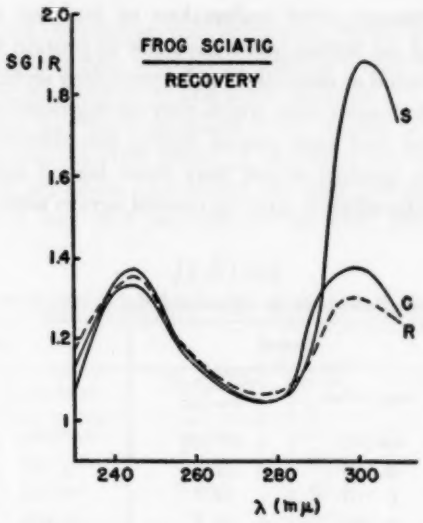

FIG. 5. Mean SGIR of frog sciatic nerve extracts from resting nerves (*C*), stimulated nerves (*S*), and stimulated nerves allowed to rest for 2 minutes (*R*) (see Table V).

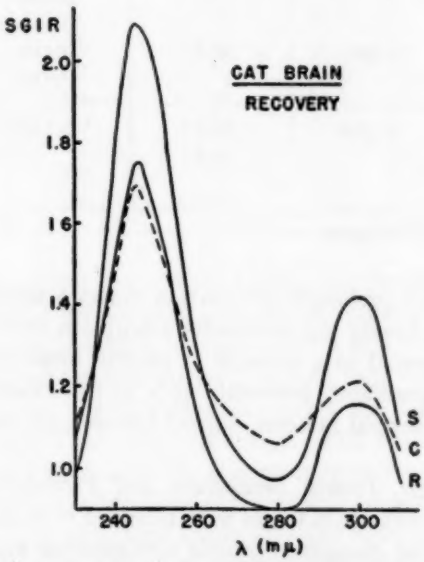

FIG. 6. Mean SGIR of cat brain extracts; from resting cortex (*C*), after stimulation of corresponding brachial plexus (*S*), and after stimulation followed by 2 minutes' rest (*R*) (see Table V).

isolated nerve, experiments were undertaken to find out whether electrical stimulation of isolated rat sciatic nerve gave rise to protein breakdown.

The nerves were treated as described in the preceding section but stimulation was maintained for 30 minutes after which they were ground up with the Ringer solution in which they had been placed during the stimulation so that any protein or breakdown product which may have leaked out from the nerve would be included in the extract. Resting control nerves were similarly treated.

TABLE VI

Protein Breakdown in Stimulated Rat Sciatic Nerve

No.	Control		Stimulated	
	Total protein	TCA-soluble fraction	Total protein	TCA-soluble fraction
	O.D./mg.	*per cent*	*O.D./mg.*	*per cent*
1	0.110	31.8	0.107	39.1
2	0.110	33.2	0.116	41.3
3	0.110	32.7	0.116	40.6
4	0.111	32.4	0.110	42.3
5	0.111	36.0	0.115	43.9
			0.105	43.8
			0.116	39.6
6	0.099	38.4	0.114	43.4
			0.110	45.5
			0.115	44.0
Mean	0.1085	33.1	0.1124	42.2
± S.D.		2.8		2.1
P				0.02–0.01

Duration of stimulus 30 minutes.

Table VI shows that prolonged stimulation causes a significant increase in acid-soluble material having the ultraviolet absorption characteristics of protein. This was interpreted as a measure of protein breakdown in the tissue. The process is not immediately reversible since, in this experiment, the nerves were not frozen and several minutes elapsed between the end of stimulation and the extraction.

5. Structural Changes, Protein Breakdown, and Proteolytic Activity of Rat Brain.—The experiments which follow were intended to study the relationship between proteolysis and changes in protein configuration and also to find out whether an enzymatic factor was involved in these processes. The presence of proteases in brain has been described by Kies and Schwimmer (1942) and by Ansell and Richter (1954). A protease was found in rat brain which acts on the synthetic substrate, N-acetyl-L-tryptophan ethyl ester. Brain extracts from

other species did not hydrolyze this substrate. Hydrolysis of the ester was measured by the manometric method as described above. Protein breakdown and the SGIR were determined in the same extracts.

As seen in Table VII brain extracts from resting rats anesthetized with nembutal (cortex removed after freezing) showed a fair amount of proteolytic activity: 10.9 μM of substrate hydrolyzed per hour per gm. of fresh tissue; the acid-soluble fraction was 14.2 per cent of the total protein. After electrical

TABLE VII

Protease Activity, Proteolysis, and SGIR in Resting Rat Brain

No.	Protease	Total protein	TCA-soluble fraction	SGIR	
				245 mμ	300 mμ
	μM/hr./gm.	*O.D./mg.*	*per cent*		
1	6.3	0.111	15.8	—	—
2	13.4	0.101	16.8	1.48	1.31
3	13.2	—	—	—	—
4	13.7	0.082	12.3	1.64	1.60
5	14.8	—	—	—	—
6	7.2	0.108	13.1	1.57	1.36
7	11.4	0.134	12.4	1.34	1.24
8	9.4	0.121	15.1	1.56	1.34
9	6.9	0.121	10.5	—	—
10	12.5	0.108	8.4	—	—
11	12.0	0.105	12.5	—	—
12	9.9	0.105	12.3	—	—
Mean.....	10.9	0.110	12.9	1.52	1.37
± S.D.....	2.9	0.014	2.5	0.11	0.14

stimulation of the central end of both sciatics for 20 minutes, the proteolytic activity increased over 100 per cent, total protein content of the brain decreased with increase of the acid-soluble fraction, and the SGIR rose (Table VIII).

When the brain was stimulated for 1 minute only (Table IX), no protein breakdown took place: the total protein content and its acid-soluble fraction remained unchanged. There was, however, a small but significant increase in protease activity and a rise in SGIR. It appears therefore that short stimulation produces only structural alterations of the protein molecule, actual breakdown taking place only with prolonged excitation. The increase in protease activity even with short stimulation suggests that configurational changes may represent the first stage of the same enzymatic process. This point will be discussed later.

6. Significance of the Side-Group Ionization Ratio.—As mentioned above, the modification in the ultraviolet absorption of alkaline solutions of proteins was explained by Crammer and Neuberger (1943) and Tanford and Roberts (1952)

by the ionization of the phenolic hydroxyl of tyrosine. In native proteins, some of these side-groups are free, others linked to neighboring peptide chains by H bonds, salt linkages, or other forces. When the protein is denatured, these bonds are ruptured and the previously bound side-groups become free and ionized. This shifts the ultraviolet absorption of tyrosine in alkaline solution

TABLE VIII

Protease Activity, Proteolysis, and SGIR in Rat Brain Stimulated for 20 Minutes

No.	Protease	Total protein	TCA-soluble fraction	SGIR	
				245 mμ	300 mμ
	μM/hr./gm.	*O.D./mg.*	*per cent*		
1	23.4	0.106	22.1	—	—
2	19.7	0.071	23.7	1.90	1.41
3	25.4	—	—	—	—
4	25.9	0.072	14.4	1.85	1.69
5	23.9	—	—	—	—
6	23.2	0.098	15.0	1.74	1.40
7	21.2	0.105	18.2	2.06	1.63
8	22.8	0.102	25.7	1.71	1.48
Mean	23.2	0.092	19.8	1.85	1.52
± S.D.	2.0	0.016	4.6	0.14	0.14
P	<0.001	0.05–0.02	0.02–0.01	0.01–0.001	0.2–0.1

TABLE IX

Protease Activity, Proteolysis, and SGIR in Rat Brain Stimulated for 1 Minute

No.	Protease	Total protein	TCA-soluble fraction	SGIR	
				245 mμ	300 mμ
	μM/hr./gm.	*O.D./mg.*	*per cent*		
1	15.6	0.112	11.1	1.74	1.42
2	19.2	0.106	8.6	1.87	1.72
3	13.8	0.113	8.3	1.90	1.54
4	14.3	0.104	14.9	1.83	1.66
5	14.6	0.094	13.9	1.87	1.65
6	19.5	0.108	12.7	1.77	1.50
7	16.7	0.115	11.2	1.78	1.43
8	18.1	0.130	11.5	1.89	1.53
Mean	16.4	0.110	11.5	1.83	1.58
± S.D.	2.1	0.009	2.3	0.06	0.10
P	0.05–0.02	—	—	<0.001	0.05–0.02
P'	<0.001	0.05–0.02	<0.001	—	—

P, compared with controls.

P', compared with 20 minute stimulation.

from 275–280 mμ to 295–300 mμ and greatly increases its extinction coefficient in the latter zone.

No reference could be found in the literature to the similar shift observed at 245 to 250 mμ. A number of amino acids were therefore examined in the ultraviolet spectrophotometer at neutral and alkaline pH. The technique was the same as that described above for the protein solutions and the nerve extracts. The concentration at which the various amino acids were examined varied with their extinction coefficient.

TABLE X

"Ionization Ratio" of Amino Acids and Some Other Compounds Present in Nerve Tissue

	Ionization ratio at		
	245 mμ	275 mμ	300 mμ
Tryptophan*	0.96	1.06	2.37
Tyrosine*	84.00	0.92	4220.00
Phenylalanine	1.47	1.56	3.27
Cysteine*	150.00	11.90	3.12
Cystine	2.11	1.35	1.82
Histidine	77.00	5.00	4.35
Lysine	1.67	0.53	0.66
Arginine	4.55	0.65	1.22
Glutamic acid	1.19	1.09	1.33
Glutamine	1.51	1.59	2.50
Serine	2.10	0.68	0.85
Hydroxyproline	5.88	1.67	2.22
ATP*	1.09	1.15	4.40
Thiamine*	5.10	3.07	93.50
5-Hydroxytryptamine*	1.88	0.89	4.88
Acetylcholine	1.79	0.97	1.21
Histamine	66.70	1.56	1.00

* $E_M > 1000$ at pH 12.

The results are listed in Table X in terms of the ratio of optical densities read at pH 12 and pH 7. It seems that two conditions must be fulfilled by an amino acid to explain the SGIR phenomenon: it has to have a high extinction coefficient at least in alkaline solution ($E_M > 1000$) and the ratio between absorptions at alkaline and neutral pH has to be at least of the order of 100. The first condition is fulfilled only by tryptophan, tyrosine, and cysteine. Tyrosine satisfies the second condition at 300 mμ (to a lesser extent at 245 mμ) and cysteine at 245 mμ. This, or course, does not mean that the side-groups of these two amino acids are the only ones to ionize in the denaturation process; but that they are the only ones which can be detected by the present method.

Table X also shows the behavior of a few compounds which, although not

amino acids, are present in nerve tissue and might conceivably play a role in the SGIR phenomenon. ATP, 5-hydroxytryptamine, and thiamine have high extinction coefficients but the ratios are too low, except for thiamine. The concentration of the latter, however, is probably not high enough for detection in the amounts of tissue used in the present experiments.

To further test the probable role of tyrosine and cysteine, varying amounts of these amino acids were added to a rat sciatic extract. Fig. 7 shows that 12.5 μg. of cysteine HCl per mg. of nerve produces a high SGIR at 245 mμ. Even less tyrosine (0.5 μg.) is required to produce a similar effect at 300 mμ.

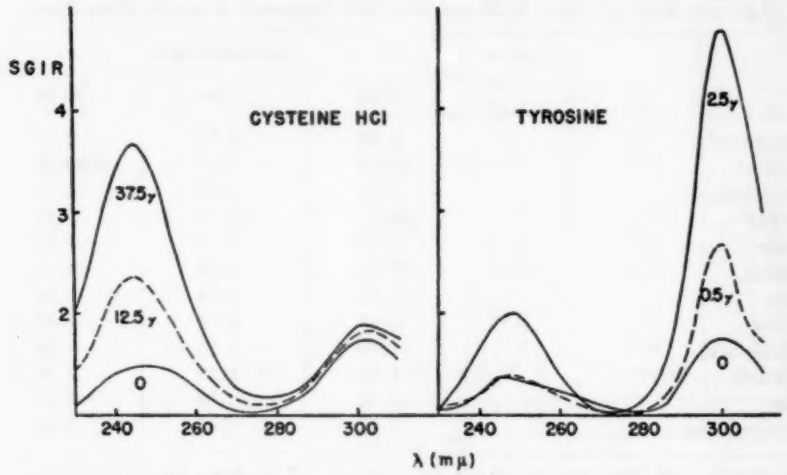

FIG. 7. Effect of the addition of cysteine or tyrosine to rat sciatic extract on the SGIR. Left, addition of cysteine HCl; right, addition of tyrosine (micrograms in 4 ml. of extract corresponding to 3 mg. of nerve).

These results suggest that the two peaks of SGIR which increase in excited nerve structures correspond to the ionization of tyrosine and cysteine. Ionization of cysteine is known to be a more complex phenomenon than that of tyrosine; it is not reversible by reacidification and probably involves oxidation of the —SH group (Steinhardt and Zaiser, 1955).

Hydrolysis of proteins would also cause increase of the SGIR. However, as was shown in rat brain, a short stimulation causes only ionization of side-chains, without detectable proteolysis.

DISCUSSION

The results reported in this paper suggest that during the state of excitation some molecular rearrangement takes place in the cell proteins. The criterion used for the estimation of this protein change would indicate that it consists essentially in an "unmasking" of amino acid side-groups which, in the native

protein, are inaccessible. It is widely assumed that these side-groups are linked together by bonds which are essential for maintaining the unique configuration of each protein. When the bonds are broken, the specific, strictly ordered configuration of the "native" protein gives place to a looser, more random structure which characterizes the "denatured" state.

There is sufficient evidence today for believing that denaturation is a reversible phenomenon and that native and reversibly denatured proteins may be present together in equilibrium (Anson and Mirsky, 1933–34; Anson, 1945). There is a great deal of indication that native and denatured forms may be different but equally "physiological" states of the same protein (Green and Neurath, 1953). It is questionable whether the term "denatured," with its original connotation of an unphysiological state, is the proper designation for a modified but still functional form of the protein molecule.

It is therefore conceivable that the stimulation causes a displacement of the equilibrium towards denaturation. With intense and prolonged stimulation, a further step may be reached with rupture of peptide bonds and breakdown of the molecule. While the first stage is readily reversible, recovery from the second stage is probably effected only by synthesis of new protein. Palladine (1956) found, by measuring the rate of incorporation of labelled methionine, an increased turnover of proteins in stimulated brain.

It is not clear how stimulation brings about the molecular rearrangement of cell proteins. Segal (1956) assumes that the first effect of the stimulus is to lower the pH, bring the proteins closer to their isoelectric point, and thus produce partial denaturation. This denaturation disrupts the coacervate which, according to Segal, is the normal state of the resting protoplasm. Part of the energy released by these processes gives rise to the electrical changes and the rest is used for the restoration of the coacervate and of the native protein configuration.

Some of the results of the present work suggest that the first stage of denaturation may be enzymatic. Linderstrøm-Lang (1952) has pointed to the probability that the first stage in the enzymatic breakdown of proteins is denaturation. Hydrolysis starts only when the enzyme can reach the peptide groups made accessible by denaturation. It is therefore theoretically possible for a protease to stop at the denaturation phase before breaking any peptide bonds ("zipper type" of Linderstrøm-Lang). Some results obtained with rat brain, reported above, seem to support this assumption.

If the mechanism of the phenomenon is enzymatic, it still remains to be found how the enzyme becomes active. There is at least one enzyme precursor, protyrosinase, which is known to be converted into the active enzyme by denaturation (Bodine, 1945). It is therefore possible that the stimulus acts first by denaturing a protease precursor and thereby activates it. The enzyme, in turn, denatures the other proteins which play a role in the excitation process.

Most denaturation reactions require energy and their ΔF is believed to be

of a very high order (Johnson *et al.*, 1954). Certain types of denaturation, however, behave differently: denaturation itself has a negative heat of reaction and it is renaturation that absorbs energy (Linderstrøm-Lang, 1952). The thermodynamic aspect of the problem is of the utmost importance because excitation must proceed by an exergonic reaction. Under physiological conditions, the energy supplied by the stimulus can only act as a trigger. It has therefore to be assumed with Segal (1956) that the net result of the denaturation caused by stimulation is release of energy and consequently renaturation requires energy.

It was seen that restoration of native protein does not take place in the nerve extract but it does occur in the intact structure if it is allowed to rest. This can probably be explained by assuming that the metabolic sources of energy required for restoration are not available in the extract.

Some physical changes observed in stimulated structures, such as increase in viscosity (Flaig, 1947) and in opacity (Tobias, 1952), are compatible with denaturation. Increased absorption of ultraviolet radiation (especially at 265 mμ) observed at the nodes of Ranvier of single myelinated neurones stimulated electrically (von Muralt, 1953), may also be indicative of change in protein structure.

One can only speculate on the connections between protein structure and concentration of ions in the cell. Meyer (1937) suggested that ionization of —COOH and —NH_2 groups in the surface proteins would control the permeability of the cell to cations and anions. In Ling's fixed charge hypothesis (1952), accumulation of K^+ in the cell depends on the preferential binding of this ion by the proteins. If the native configuration of these proteins breaks down, the fixed charges can be occupied indiscriminately by Na^+ or K^+ and the net result would be loss of K^+ and gain of Na^+ by the cell. Restoration of the native state would reverse the process. On the whole, such a system has a great deal in common with the "pump" hypothesis. If Na^+ is kept out of the cell because it cannot be accommodated by the native proteins, the metabolic process which maintains and restores the native configuration can be called a "sodium pump," without unduly stretching the point.

Mirsky (1936) has suggested that the bleaching of visual purple has many of the properties of reversible denaturation. Wald and Brown (1951–52) have followed the effect of light on rhodopsin by amperometric titration of the —SH groups and were impressed by the fact that the unmasking of these groups gave rise to measurable electrical variations. It remains to be seen whether similar results would be obtained if the Ag^+ of the amperometric system were replaced by physiological ions. It is also a matter of conjecture whether the sulfhydryl groups play a special role, as also claimed by Kostoyanc (1956), or whether other ionized groups can take part in the phenomenon.

The anology with rhodopsin also suggests the possibility that the "excitable" protein is linked to some non-protein moiety (*e.g.* phospholipids) which breaks off when the protein is denatured by stimulation. Such a possibility is supported by a number of unconnected observations (Lillie, 1922; Folch and Lees, 1951; Geiger *et al.*, 1956).

The results reported in this paper indicate that stimulation causes structural changes in proteins. However, the role of this phenomenon in the mechanism of excitation depends on the possibility that denaturation and renaturation can take place within the temporal limits of a single nerve impulse. In the meantime, it may be premature for a "denaturation hypothesis" to compete with the membrane theory for the explanation of the mechanism of excitation.

SUMMARY

Changes in the configuration of proteins were studied by the modifications of the ultraviolet absorption of their alkaline solutions. These were expressed in terms of the ratio $O.D._{pH12}/O.D._{pH7}$, termed side-group ionization ratio (SGIR). This ratio showed two peaks; one at 300 to 305 mμ is known to correspond to the phenolic hydroxyl of tyrosine and another at 245 mμ seems to be caused by the ionization of the sulfhydryl group of cysteine.

The SGIR of extracts from electrically stimulated nerve structures was found to be consistently and significantly higher than that of similar extracts from resting tissues. The phenomenon was observed in isolated nerves (frog and rat sciatic) stimulated *in vitro* and in the cerebral cortex of cats, dogs, and rats after stimulation of their afferents. The increase in SGIR was reversible if the stimulated structures were allowed to rest.

Prolonged stimulation, in addition to causing structural changes, also caused breakdown of proteins and the appearance of proteolytic activity. The latter, studied on a synthetic substrate, could be detected even after shorter stimuli, together with configurational changes but without proteolysis.

The structural changes detected with the spectrophotometric method are closely related to reversible denaturation as produced by urea. The changes probably involve rupture of hydrogen bonds which loosens the protein molecule and perhaps changes its affinity for different ions. It is possible that such a process may play a role in the mechanism of excitation.

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THE EFFECT OF ULTRAVIOLET AND WHITE LIGHT ON GROWTH RATE, LYSIS, AND PHAGE PRODUCTION OF BACILLUS MEGATHERIUM

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(Received for publication, January 12, 1957)

SUMMARY

Cultures of *megatherium* 899*a*, growing under different conditions, were exposed to ultraviolet or white light.

1. Cultures exposed to ultraviolet light and then to white light continue to grow at the normal rate. Cultures exposed to ultraviolet light and then placed in the dark grow at the normal rate for varying lengths of time, depending on conditions, and then lyse with the liberation of from 5 to 1000 phage particles per cell, depending on the culture medium.

2. Increasing the time of exposure to ultraviolet light results in an increase in the fraction of cells which lyse in the dark. The lysis time decreases at first, remains constant over a wide range of exposure, and then increases. The lysis can be prevented by visible light after short exposure, but not after long exposures.

3. The time required for lysis is independent of the cell concentration.

4. *Effect of temperature.* After exposure to ultraviolet the cell concentration increases about 4 times at 20°, 30°, or 35°C., but only 1.5 to 2.0 times at 40–45°. This is due to the fact that the growth rate of the culture reaches a maximum at 38° while the lysis rate increases steadily up to 45°.

5. Terramycin decreases the growth rate and lysis rate in proportion.

6. At pH 5.1, the cultures continue to grow slowly in the dark after exposure to ultraviolet light.

7. *Megatherium* sensitive cells infected with T phage lyse more rapidly than ultraviolet-treated 899*a*, and visible light does not affect the lysis time.

The results agree with the assumption that exposure to ultraviolet results in the production of a toxic (mutagenic) substance inside the bacterial cell. This substance is inactivated by white light.

The Effect of Ultraviolet and White Light on Growth Rate, Lysis, and Phage Production of B. Megatherium

Exposure of organisms to ultraviolet light results in an increase in the proportion of mutants and eventually in the death of the organism. Exposure of

organisms to ultraviolet is much more effective, if the organisms are kept subsequently in the dark, than if they are exposed to intense white light (The subject has been critically reviewed by Muller, 1954.)

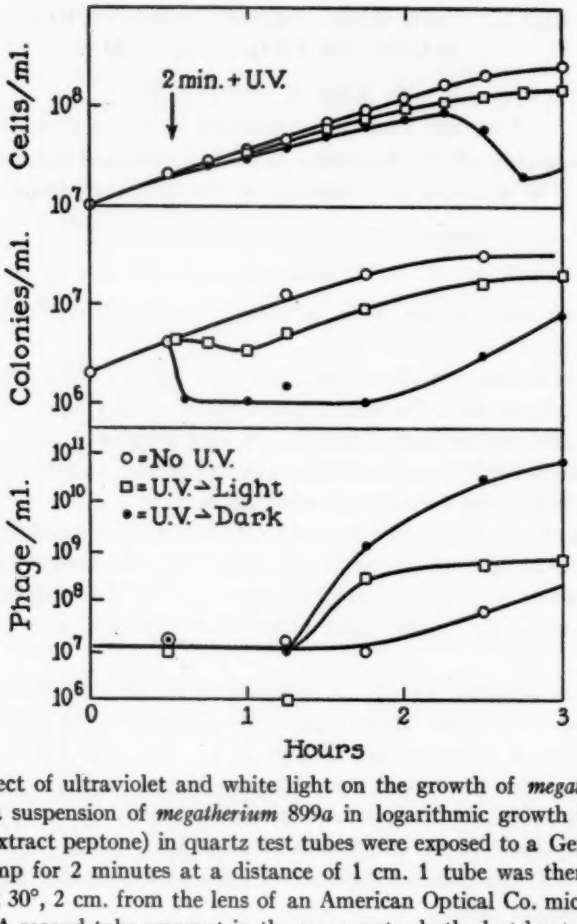

FIG. 1. Effect of ultraviolet and white light on the growth of *megatherium* 899*a*. 10 ml. of a suspension of *megatherium* 899*a* in logarithmic growth in 5 per cent YEP (yeast extract peptone) in quartz test tubes were exposed to a General Electric germicidal lamp for 2 minutes at a distance of 1 cm. 1 tube was then placed in a water bath at 30°, 2 cm. from the lens of an American Optical Co. microscope lamp (model 370). A second tube was put in the same water bath, but kept dark. A third tube was not exposed to ultraviolet. All tubes were stirred by bubbling air.

The white light may be assumed to reanimate cells which have been killed by the ultraviolet light ("photoreactivation"), or to destroy some toxic compound formed by the ultraviolet light. Novick and Szilard (1949) have shown that this latter assumption explains the facts very well, and hence, it is unnecessary to suppose that the visible light reanimates cells killed by the ultraviolet.

If lysogenic cultures are exposed to ultraviolet, they grow for some time

and then lyse (Lwoff, Siminovitch, and Kjeldgaard, 1950). This process may be prevented by visible light (Jacob, 1950). In the case of lysogenic cultures, therefore, the number of living and growing cells may be determined by direct observation, since the dead cells disintegrate. The results of an experiment carried out in this way are shown in Fig. 1. The figure shows that both cultures exposed to ultraviolet grow at very nearly the same rate as the control for 2 hours, whether they are in white light or not. At the end of this time, the cells in the dark tube lyse with the liberation of about 1000 phage particles per cell. The culture placed in the light shows only about 10 per cent lysis.

It is evident that the colony counts from these cultures are entirely misleading. The colony count of the dark culture, for instance, drops to less than 10 per cent of the original value immediately after the ultraviolet treatment, and yet the culture from which the sample was taken continues to grow at the normal rate. The colony count of the culture in the light is also only about half that of the control; yet this culture continues to grow indefinitely. These paradoxical results are due to the fact that the colony counts of the cultures are incorrectly placed on the time axis. The colony count immediately after ultraviolet exposure, for instance, represents those cells which were able to survive 2 minutes of ultraviolet and also 8 to 10 hours in the dark on the agar plate. It does not represent the number of viable cells immediately after the ultraviolet exposure, and, in fact, it is impossible to place the colony count on the time axis without further information. The difficulty cannot be avoided by defining viable cells as those capable of forming colonies, since it is still not possible to say when the ability to form colonies is lost.

If, on the other hand, the injury is due to some toxic (mutagenic) substance formed in the cell by the ultraviolet and inactivated by light, then the results are to be expected. This mechanism also explains the effect of temperature on the reaction. Cultures exposed to ultraviolet and then kept in the dark at 15° retain the ability to grow, when exposed to light, about four times as long as those kept at 35°. The inactivation reaction, therefore, has a high temperature coefficient. This is not true of photochemical reactions (Novick and Szilard, 1949; Giese *et al.*, 1956).

Effect of Temperature, Light, pH, Terramycin Concentration, and Cell Concentration on Growth and Lysis of Megatherium 899a after Exposure to Ultraviolet. Table I

Time of Exposure to Ultraviolet.—Increasing the time of exposure to ultraviolet increases the lysis until it becomes complete. The lysis time remains constant, at first, and then increases on long exposure. Lysis after long exposure cannot be prevented by visible light. This result indicates the existence of two reactions. The toxic product formed in the cell by the ultraviolet alters some essential cell constituent (probably DNA) resulting in a mutation. Very

little toxic agent is sufficient to carry out this reaction, and an excess does no more damage. Hence the percentage of lysis and the lysis time remain constant

TABLE I

Effect of Temperature, Light, pH, Terramycin Concentration, and Cell Concentration on the Growth Rate and Lysis Time of megatherium 899a after Exposure to Ultraviolet Light

Culture media and *megatherium* strain	Length of exposure to UV	Temp.	pH	B_0	Growth	Per cent lysis		Lysis time		B_{max}/B_0		Phage particles per cell
						Dark	Light	Dark	Light	Dark	Light	
	(1 cm.)				*rate/hr*			*hrs.*	*hrs.*			
YEP	0	35	6.8	1×10^7	1.8	0	0					
Megatherium 899*a*	1 min.				1.8	50	10	1.3		3.5		
	2				1.7	100	10	1.3		3.0		
	5				1.1	100	10	1.5		3.0		1,000
	10				0.1	100	80	2.2	2.0	2.5	2.5	
	2	35		1×10^6				1.3				
				1×10^4				1.2				
				1×10^2				1.2				
0.5 γ terramycin/ml.	2	35		1×10^7	0.7	100		2.1		3.8		
YEP	0		5.1		0.1–1.0	0						
	2		5.1		0.1–1.0	10						
	2	40	6.8		1.9	100	10	1.1		3.0		
	2	44			1.7	100	10	0.8		2.0		
	2	30			1.3	100	10	2.5				
	2	25			1.0	100	10	2.0		4.0		
	2	20			0.75	100	10	4.4		4.0		
Asparatic acid–arginine	(10 cm.)											
10^{-3} M $Mg.^{++}$	10 sec.	35			1.0	80		1.5		2.5		100
10^{-5} M $Mg.^{++}$	10 sec.	35			0.3	80		1.5		1.5		5
	(10 cm.)											
YEP	0	40			1.8	0	0					
Megatherium sensitive	15 min.	40			1.0	0	0					
Megatherium sensitive + T phage	0	40			1.0	100	100	0.6	1.7	1.7		500

for a wide range of ultraviolet exposure. (This is the usual effect of ultraviolet on the mutation rate.) Further exposure injures the metabolism of the cell and eventually kills it, possibly through direct action of the ultraviolet light, since this effect is not reversible by visible light. This explanation is partly sub-

stantiated by the fact that the sensitive strain of *B. megatherium* does not lyse after short exposure, but is killed after long exposure. The failure of such strains to lyse is due to the fact that ultraviolet and mutagenic agents, in general, merely increase the proportion of mutants already present, rather than cause new ones to appear (Muller, 1954). There are no lysogenic mutants in cultures of the sensitive strain, and, hence, exposure to ultraviolet cannot increase them.

Effect of Time of Exposure to White Light.—Under the conditions used in these experiments, 20 minute exposure to white light decreased lysis to about half and 40 minutes prevented practically all lysis.

Effect of Cell Concentration.—The percentage of lysis and the time required for lysis after 2 minutes of exposure to ultraviolet is practically independent

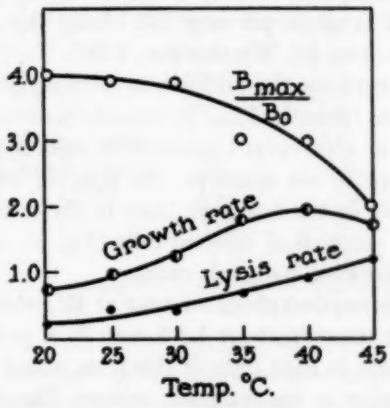

FIG. 2. Effect of temperature on the growth rate, lysis rate, and ratio of B_{max}/B_o. 899a in logarithmic growth in 5 per cent YEP exposed to ultraviolet for 2 minutes at 1 cm. Culture then grown at temperature noted in the dark.

of the cell concentration. If anything, the dilute suspension lyses first. These experiments were done by exposing a suspension of 1×10^8 B/ml. to ultraviolet for 2 minutes and then diluting to 1×10^4 and 1×10^2 B/ml. at once. The suspensions were all shaken at 35° and plated for phage at 15 minute intervals. No damage had been done to the cells at the time the dilution was made, since they grew normally if placed in the light. If the toxic product formed by the light were present in the culture medium, then it would be expected that the more concentrated suspension would lyse first. Since lysis occurs at about the same time in the concentrated suspension as in the one which had been diluted $1/10^6$, the toxic substance is probably *in* the cell (Roberts and Aldous, 1949). This may explain why the preventive effect of light does not appear in molecular reactions, since in these the concentration of reactants is very low, while inside a cell it may be very high. Effects similar to those ob-

served with cells might be expected with very concentrated (>30 per cent) solutions of nucleic acids, but not with dilute ones. It may be for this reason that light has no effect on ultraviolet-inactivated phage particles.

Effect of Temperature on Growth Rate, Growth before Lysis, and Lysis Time

The effect of various temperatures on the growth rate, the growth before lysis occurs, and the lysis rate ($1/t$ to cause lysis) is shown in Fig. 2. The lysis rate has a steady temperature coefficient of about 2 per 10 degrees from 20-45°, while the growth rate, as usual, shows a maximum near 40°. As a result, the ratio B at lysis/B at beginning decreases as the temperature increases. Lysis is not conditioned, therefore, by a certain number of cell divisions, but rather by a certain time limit.

The growth rate and lysis rate may be separated by growing the exposed cells in a very narrow range of pH near 5.0. Under this condition, very slow growth occurs, but no lysis (*cf.* Weatherwax, 1956).

If the growth is slowed by the addition of terramycin, however, the lysis time is also slowed, and the ratio B_{max}/B_o remains constant.

The time for lysis of ultraviolet-treated 899*a* cells is longer than that required for lysis of *megatherium* sensitive cells infected with T phage and the ratio B_{max}/B_o is higher in the lysogenic than in the infected system.[1] White light has no effect on the lysis of infected cells (Fig. 3), nor does it affect the phage production in growing lysogenic cultures.

Lysis of infected cells requires about 0.6 hour at 35°, while lysis of ultraviolet-treated lysogenic cells requires about 1.3 hours. It is probable that the lytic process itself is the same in both cells; if this is so, about 0.7 hour is required to start phage production in the lysogenic culture. The results in Fig. 3 also show that the lytic process itself is not affected by white light and hence, the light should not prevent lysis unless it is used at least half an hour before lysis would occur. This is approximately correct. Ultraviolet-treated cells will grow normally if exposed to light ½ hour after the ultraviolet (at 35°) but not after 1 hour.

Effect of Culture Media.—In synthetic medium made up of aspartic acid, arginine, glucose, sodium and potassium phosphate, iron and magnesium sulfate, the culture is much more sensitive to ultraviolet light. This is due largely to the fact that this solution does not absorb ultraviolet as strongly as the peptone. The lysis time after exposure to ultraviolet is about the same as in peptone, but the growth and the phage yield per cell is less in 10^{-3} M $MgSO_4$ and much less in 10^{-5} M $MgSO_4$ than in peptone. The lower phage yield is no doubt due to the lower NA content of slow growing cells (Krueger and Mundell, 1938; Hedén, 1951; Northrop, 1953), since the cell NA is the precursor of phage NA (Hershey, 1953).

[1] *Megatherium* sensitive cells infected with C phage in very dilute suspensions, however, go through 2 or 3 cell divisions before lysis occurs (Northrop, 1953).

The increased phage yield in the presence of $MgSO_4$ (Northrop, 1951) is probably due to an increase in phage particles produced per cell rather than an increase in the number of phage-producing cells, as is the case when the culture is exposed to ultraviolet.

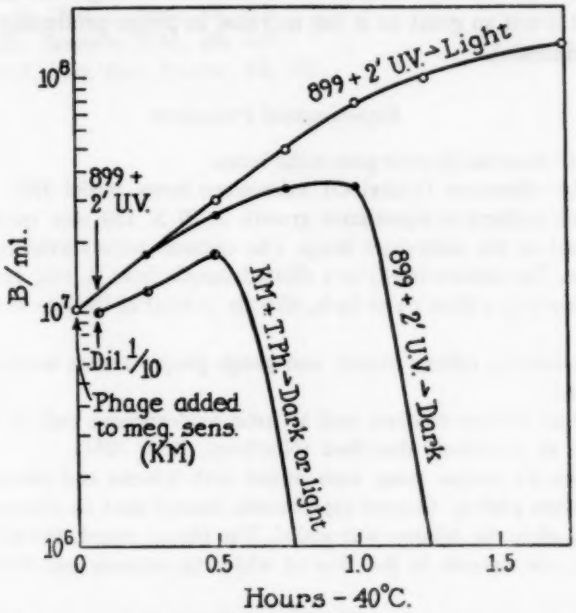

FIG. 3. Effect of white light on *megatherium* 899*a* after exposure to ultraviolet and on *megatherium* sensitive, after infection with T phage.

899*a* in logarithmic growth in YEP. 1×10^7 B/ml. in quartz test tubes. Exposed to ultraviolet for 2 minutes at 1 cm. and then grown in dark or light at 40°.

Megatherium sensitive in logarithmic growth in YEP. 1×10^8 cells/ml. 1×10^{10} phage particles/ml. added, the suspension shaken at 25° for 5 minutes and then diluted $\frac{1}{10}$ in YEP. One tube in white light and 1 tube dark at 40°C. Colony counts made at the time of dilution showed that more than 90 per cent of the cells were infected. The increase in the cell count of the infected culture, therefore, cannot be ascribed to the presence of uninfected cells.

Effect of Ultraviolet on the Mutation Rate of Terramycin-Resistant Mutants, and of Phage-Producing Cells

The effect of ultraviolet and white light (or other mutagenic agents) on phage production of lysogenic cultures is similar in all respects to the effect on the mutation rate of various bacterial mutants. This suggests the probability that the change from a normal cell to a phage-producing cell is also a mutation.

This explanation is suggested also by the fact that the phage particle is a product of cell metabolism (Bordet, 1931; Northrop, 1938; Lwoff, 1954; Raet-

tig, 1955; Northrop and Murphy, 1956; Welsch, 1956) and its production is a genetic character of the bacterial cell, just as is the production of the transforming principle (Gratia, 1936; Fredericq, 1953; Lederberg and Lederberg, 1953; Wollman, 1953). Exposure to ultraviolet light does, in fact, increase the mutation rate of terramycin-resistant mutants in *B. megatherium* cultures, but the increase is not so great as is the increase in phage-producing cells (unpublished experiments).

Experimental Procedure

Ultraviolet—General Electric germicidal lamp.

White light—American Optical Co. microscope lamp, model 370.

10 to 15 ml. cultures in logarithmic growth in 20 × 150 mm. quartz tubes were placed in front of the ultraviolet lamp. The cultures were stirred by bubbling air through them. The culture itself, or a diluted sample from it, was exposed to white light by placing it in a glass water bath, directly in front of the lens of the microscope lamp.

Cell concentration, colony counts, and phage plaque counts were made as previously described.

Yeast extract culture medium and aspartic acid–arginine culture medium were also prepared as previously described (Northrop, 1957; 1951).

The samples for plaque assay were mixed with toluene and allowed to stand 2 to 3 hours before plating. Control experiments showed that no change in the phage titer occurred after the toluene was added. The plaque count, therefore, unlike the colony count, corresponds to the time at which the sample was taken.

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STUDIES ON THE ELECTRICAL PROPERTIES OF A SINGLE PLANT CELL

Internodal Cell of Nitella flexilis

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(Received for publication, July 30, 1956)

There is a close relationship between the electrical properties of a cell and its physiological activity. By measuring the impedance of the cell we can follow the changes in its electrical properties continuously. Many studies have been done along this line and have contributed a great deal to our knowledge of cell physiology. The author intends, in this report, to introduce a new method of analysis, with which changes in the resistance of the protoplasmic membrane and of the cell sap of a *Nitella* cell can be studied simultaneously.

Material

The internodal cells of *Nitella flexilis* are generally 3 to 5 cm. long (sometimes reach above 10 cm.) and 0.4 to 0.5 mm. in diameter. This size and shape are very suitable for studying electrical characteristics of the cell.

A large part of the cell volume is occupied by a vacuole which is surrounded by a thin layer of protoplasm of about 10 μ thick and by a cellulose wall also about 10 μ thick. The inner and outer membranes of this protoplasmic layer have a higher resistance than the protoplasmic bulk between these boundary membranes, and are considered to be selectively permeable to ions. For convenience, we call the sum of the transverse resistances of the outer protoplasmic membrane, protoplasmic layer, and inner protoplasmic membrane the resistance of the protoplasmic membrane.[1] According to the measurements in moist chamber, this resistance is generally 250 kΩ cm. or more (Blinks, 1930). The protoplasmic membrane has a capacity about 1.0 μf./cm.^{2} (Blinks, 1930; Curtis and Cole, 1937; Cole and Curtis, 1950) and the specific resistance of cell sap is about 60 Ω cm. (Curtis and Cole, 1937). Although these resistances change when the constituents or concentrations of the outer medium are varied, the phase angle of the impedance of the protoplasmic membrane is considered to remain nearly constant; *i. e.*, 80° (Curtis and Cole, 1937; Cole and Curtis, 1950).

[1] Greater part of change in this resistance may be attributed to the resistances of inner and outer protoplasmic membrane. At present, however, we do not know whether it comes from the inner membrane or outer membrane or both.

Apparatus

Measuring vessels which have two or three pools are made of paraffin. Each pool has a platinized platinum electrode as shown in Fig. 1. Between these pools grooves of 2 mm. in width and 4 mm. in depth are cut in line. An internodal cell is mounted in this vessel through these grooves where the cell is

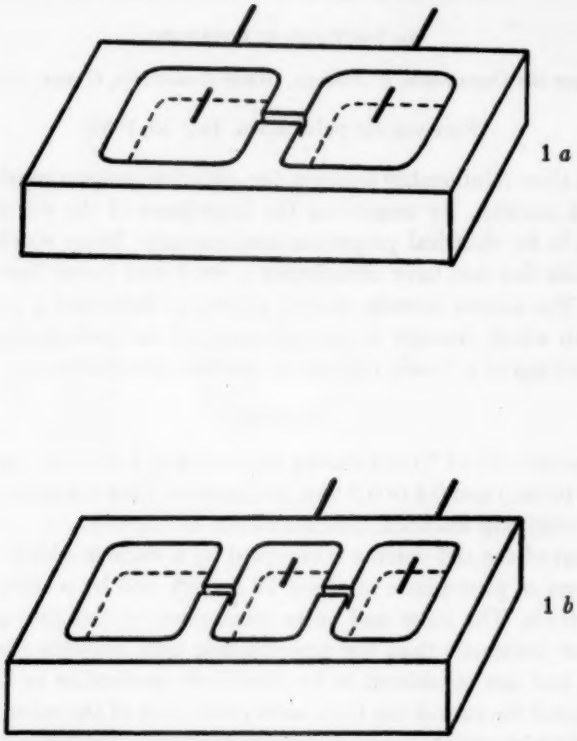


FIG. 1 *a*, 1 *b*. Vessels for measuring the impedance of an internodal cell of *Nitella flexilis*.

sealed with white vaseline. The cell can be separated in two or three parts by this procedure. Each pool is generally filled with 10^{-2} M KCl solution as a standard solution and most chemicals are dissolved in this solution. The vessel shown in Fig. 1 *a* is used for studying the effect of chemicals on the whole cell, while the vessel shown in Fig. 1 *b* is used for studying the effect of chemicals on one part of the cell. Internodal cells are placed in 10^{-2} M KCl solution at least 3 hours in advance and allowed to equilibrate before being used for measurements.

Impedances were measured with the usual bridge method as shown diagrammatically in Fig. 2. A frequency of 1000 cycles per second was supplied

with an audio-frequency oscillator, with the voltage less than 10 mv., which produced no detectable injury in the cell. The output of the bridge circuit was amplified by a four stage amplifier and led to a cathode ray oscilloscope which was used only as a null indicator.

Method of Analysis

It is necessary to estimate the magnitude of possible errors beforehand in order to make measurements with such a procedure. To know the magnitude of electrical leakage along the cell in the groove, we must estimate the magnitude of longitudinal resistances of the cellulose wall and of the protoplasmic layer. After cutting the cell

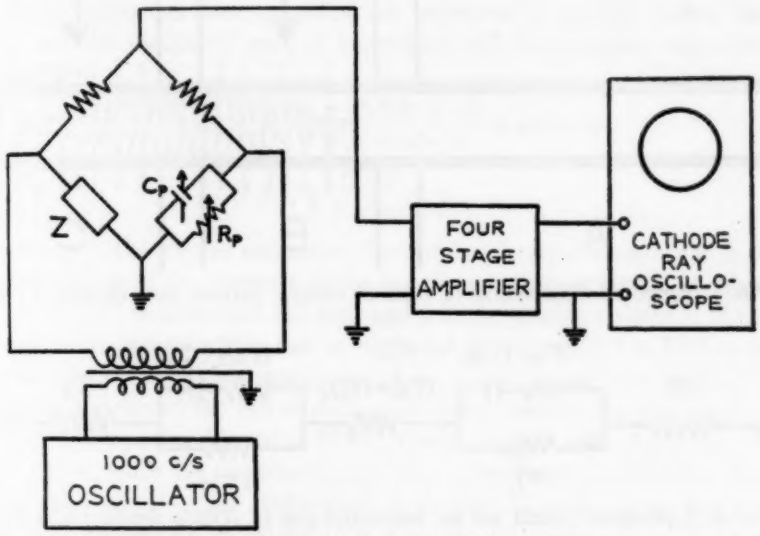


FIG. 2. Diagrammatic representation of the circuit for impedance measurements.

and squeezing out most constituents, *i. e.* protoplasm and cell sap, we can get a cellulose wall sheath. The resistance of this sheath measured in the vessel with 10^{-2} M KCl as the external medium amounts to $3 \times 10^6\ \Omega$ or more per cm. Although we have only few reliable data about the specific resistance of protoplasm, it can be estimated at 300 Ω cm. or more (Gelfan, 1928). Taking 10 μ as the thickness of protoplasmic layer, the resistance of protoplasmic layer along the cell amounts to $2.6 \times 10^6\ \Omega$ or more per cm. Referring to the value of impedance $Z_R = 5 \times 10^4\ \Omega$ as will be shown later, the sum of the leakages along the cellulose wall and the protoplasmic layer is small enough to be neglected.

We adopted 10^{-2} M KCl as a standard solution for the following reasons: (*a*) the resistance of the external solution is very small (a few hundred ohms) and therefore its possible change which might be ascribed to the ionic leakage from the cell is negligibly small when compared to $Z_R = 5 \times 10^4\ \Omega$. (*b*) With such a concentration, the potential difference between external medium and

cell interior is almost null (Osterhout, 1935). Therefore we can avoid any possible errors due to the local potential changes; *i. e.*, action potential, etc.

From these considerations, the lines of current flow are assumed to pass through the cell as shown in Fig. 3. Hence an equivalent circuit as shown in Fig. 4 can be taken.

Since the length of a cell differs generally in each pool, its contribution to the impedance also differs in each pool. Therefore it is necessary to distinguish these by adding suffix 1 and 2 to them. ρ_1 and ρ_2 are the resistances of external solutions, $(r)_1$ and $(r)_2$ are the resistances of protoplasmic membrane, and

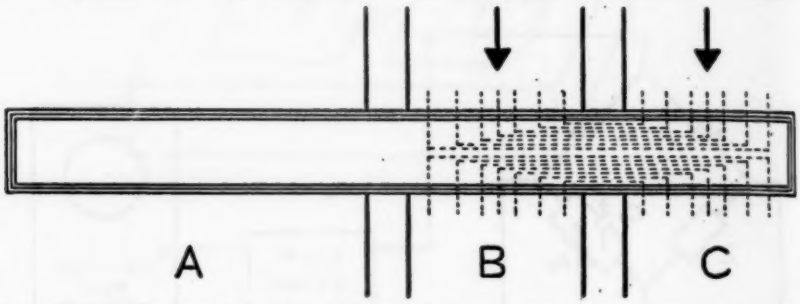


FIG. 3. Supposed distribution of lines of electric current flow through a cell.

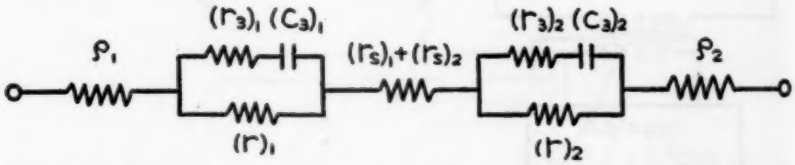


FIG. 4. Equivalent circuit for an internodal cell of *Nitella flexilis* under experimental arrangement as shown in Fig. 1.

$(r_s)_1$ and $(r_s)_2$ are the resistances of cell sap. These are all direct current resistances and do not change their values even if the frequency of alternating current is varied. On the other hand, $(r_3)_1$, $(r_3)_2$, $(c_3)_1$, and $(c_3)_2$ are the elements of proper impedance of the protoplasmic membrane and are related to its molecular structure. These have definite values under a constant frequency, although they decrease with increasing frequency (Fricke, 1932). Then the total impedance of this equivalent circuit can be calculated with the following equations:

$$Z = Z_R - jZ_X \tag{1}$$

$$Z_R = (r)_1 \cdot \left[1 - \frac{(r)_1\{(r)_1 + (r_3)_1\}\omega^2(c_3)_1^2}{1 + \omega^2(c_3)_1^2\{(r)_1 + (r_3)_1\}^2}\right] + (r)_2 \cdot \left[1 - \frac{(r)_2 \cdot \{(r)_2 + (r_3)_2\}\omega^2(c_3)_2^2}{1 + \omega^2(c_3)_2^2\{(r)_2 + (r_3)_2\}^2}\right] + (r_s)_1 + (r_s)_2 + \rho_1 + \rho_2 \tag{2}$$

$$Z_X = \frac{(r)_1 \cdot \{\omega(c_3)_1 \cdot (r)_1\}}{1 + \omega^2(c_3)_1^2\{(r)_1 + (r_3)_1\}^2} + \frac{(r)_2 \cdot \{\omega(c_3)_2 \cdot (r)_2\}}{1 + \omega^2(c_3)_2^2\{(r)_2 + (r_3)_2\}^2} \tag{3}$$

in which ω is 2π times the frequency of applied voltage and j means the square root of -1. These impedances are to be compared with the measured parallel resistance R_p and parallel capacity C_p (Fig. 2) by using equations (4) and (5).

$$Z_R = \frac{R_p}{1 + \omega^2 C_p^2 R_p^2} \tag{4}$$

$$Z_X = \frac{R_p(\omega C_p R_p)}{1 + \omega^2 C_p^2 R_p^2} \tag{5}$$

When resistances and capacities are expressed in specific values, the real part and the imaginary part of impedance will be rewritten respectively as follows:—

$$Z_R = p \cdot \frac{r\{1 + \omega^2 c_3^2 r_3(r + r_3)\}}{1 + (r + r_3)^2\omega^2 c_3^2} + mr_s + q\rho \tag{6}$$

$$Z_X = p \cdot \frac{r^2(\omega c_3)}{1 + (r + r_3)^2\omega^2 c_3^2} \tag{7}$$

in which r is the specific resistance of protoplasmic membrane, r_3 and c_3 are the specific values of the elements of the impedance of protoplasmic membrane, r_s is the specific resistance of cell sap, and ρ is the specific resistance of external solution, the last of which can be neglected if we use 10^{-2} M KCl as the external solution as in this experiment. p, m, and q are the constants which depend on the size of the cell in each pool. If we measure the impedance of the cell with alternating current of 1000 c/s only, the real part, Z_R, is the function of r and r_s, while the imaginary part, Z_X, is the function of r only. These relationships may be well understood if we plot Z_R and Z_X against r taking $pr_3 = 25$ $k\Omega$ and $mr_s = 30$ $k\Omega$[2] (Fig. 5).

Assuming $(\omega c_3 r_3)^2 \ll 1$, which was justified by Curtis and Cole (1937) and also ascertained by the author,[3] the following approximations will be taken:

$$Z_R = pr_3 + mr_s \qquad (r \geqq r_3) \tag{8}$$

$$= pr + mr_s \qquad (r \ll r_3) \tag{9}$$

Therefore we can get the following equations:

$$\frac{\Delta r_s}{r_s} = \frac{\Delta Z_R}{Z_R - pr_3} \qquad (r \geqq r_3) \tag{10}$$

$$= \frac{\Delta Z_R - p\Delta r}{Z_R - pr} \qquad (r \ll r_3) \tag{11}$$

---

[2] pr_3 and mr_s cannot be determined without knowing exact values of r_3, r_s, p, and q. Referring to the measured values of impedances Z_R and Z_X and to the experiment on osmosis, however, these values can be regarded as appropriate ones.

[3] Phase angle of the membrane impendance is about 72° in this experiment when the cell is in 10^{-2} M KCl solution.

When Z_R is 50 kΩ or more, it is nearly constant against changes in the resistance of the protoplasmic membrane (r). In other words, Z_R can be regarded as the function of r_3 only (Equation 10). But Z_R depends also on the resistance of the protoplasmic membrane in the case when the latter decreases considerably (Equation 11, Fig. 5).

On the other hand, the relation between Z_X and r is shown as follows:—

$$r = \sqrt{\frac{1}{\omega c_3} \cdot \frac{Z_X}{p - \omega c_3 Z_X}} \qquad (r \geqq r_3) \quad (12)$$

$$= \sqrt{\frac{Z_X}{p\omega c_3}} \qquad (r \ll r_3) \quad (13)$$

Therefore we can get the following two equations:[4]

$$\frac{\Delta r}{r} = \frac{1}{2} \cdot \frac{1}{1 - \frac{\omega c_3}{p} \cdot Z_X} \cdot \frac{\Delta Z_X}{Z_X} \qquad (r \geqq r_3) \quad (14)$$

$$= \sqrt{1 + \frac{\Delta Z_X}{Z_X}} - 1 \qquad (r \ll r_3) \quad (15)$$

Since Z_R and Z_X can be calculated from the measured R_p and C_p with equations (4) and (5), we can calculate the changes in the resistance of cell sap and protoplasmic membrane with equations (10), (11), (14), and (15) when the cell is subjected to different physiological situations.

RESULTS

To provide experimental evidence in support of the analysis introduced above, experiments were done first on the problem of osmosis. Later, the effects of some fat solvents on the cell were studied as an application of the method.

Osmosis (*Transcellular*).—When a cell is divided into two parts with a vessel as shown in Fig. 1 and the concentration of the external solution around one part is made osmotically higher with sucrose than around the other part, water enters the cell at the water side, passes along inside the cell, and escapes at the sucrose side (Osterhout, 1949; Kamiya and Tazawa, 1956). Being carried by water, ions and molecules are observed to accumulate in the vacuole at the sucrose side (Kamiya and Kuroda, 1956). Therefore resistance changes in the cell sap and possibly in the protoplasmic membrane on both sides of the cell

[4] $\omega c_3/p$ in Equation 14 is $1.26 \times 10^{-8} \cdot \Omega^{-1}$, if we take $pr_3 = 25$ kΩ and $\omega c_3 r_3 = 1/\lg 72° = 0.316$. This equation can be applied when $\Delta Z_X/Z_X \ll 1$. If Z_X changes greatly, the following equation should be used.

$$\frac{\Delta r}{r} = \left(1 + \frac{\Delta Z_X}{Z_X}\right)^{\frac{1}{2}} \cdot \left(1 - \frac{\omega c_3 \Delta Z_X}{p - \omega c_3 Z_X}\right)^{-\frac{1}{2}} - 1.$$

are to be expected. During this process the volume changes of the cell at both sides are, if any, small enough to be neglected.

This process, "transcellular osmosis," was observed with different concentrations of sucrose, *i. e.* 0.1, 0.2, 0.3, 0.4, and 0.5 M in 10^{-2} M KCl, the results of which are shown in Fig. 6*a*, *b*, *c*, *d*, and *e*. A sucrose solution of 0.3 M or more is concentrated enough to induce plasmolysis if applied to the whole cell, the osmotic pressure of which corresponds to 0.29 M sucrose. However, it does not

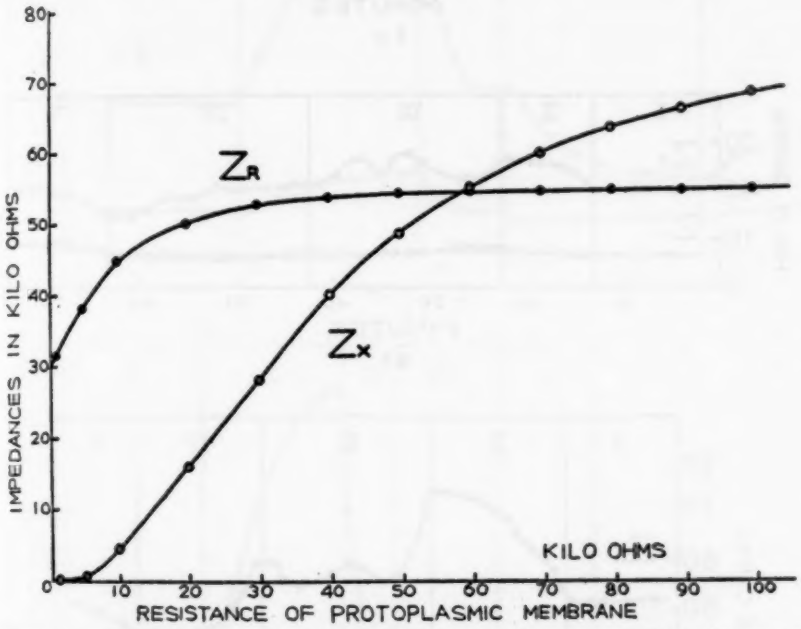
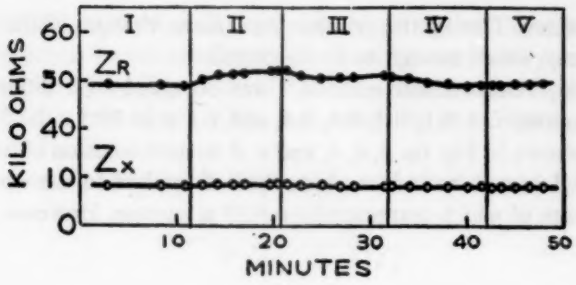
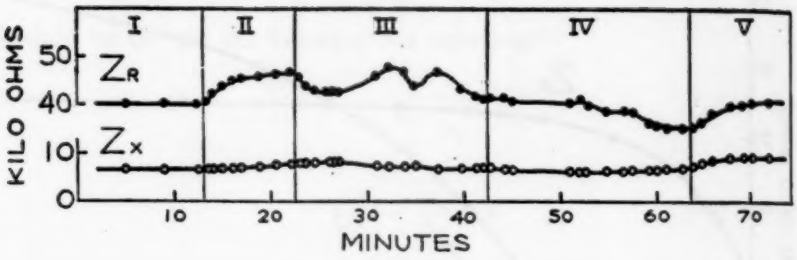


FIG. 5. Impedances, Z_R and Z_X, which are expressed with equations (6) and (7) are plotted against the resistance of the protoplasmic membrane, r. Parameters, pr_1 and mr_m, are taken 25 kΩ and 30 kΩ respectively.

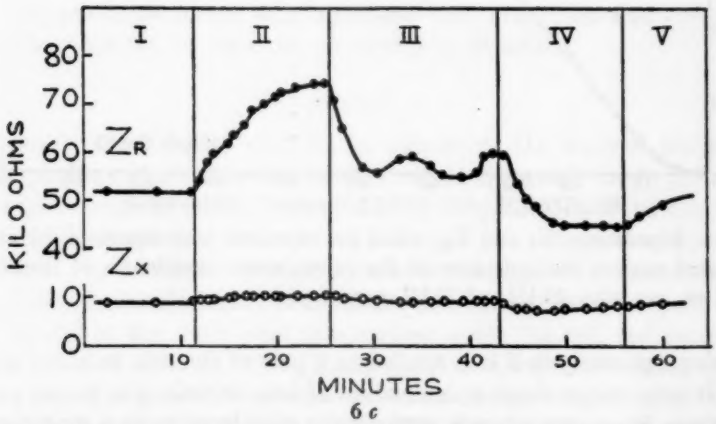
bring about plasmolysis if it is applied to a part of the cell, as in our experiment. It only brings about accumulation of ions or molecules in one part of the vacuole. Since protoplasmic streaming is considered to be a good measure of the vitality of plant cells, observations on this were made during the measurements. The protoplasm keeps on streaming under transcellular osmosis with sucrose solutions as high as 0.5 M, but it is no longer as vigorous as with lower concentrations. It recovers its vigorous streaming if the external medium is replaced with the initial 10^{-2} M KCl. The phenomena such as the contraction of chloroplasts or formation of "splits" in the chloroplast layer, as reported by Osterhout (1948), were not found in the process of transcellular osmosis.



6 *a*



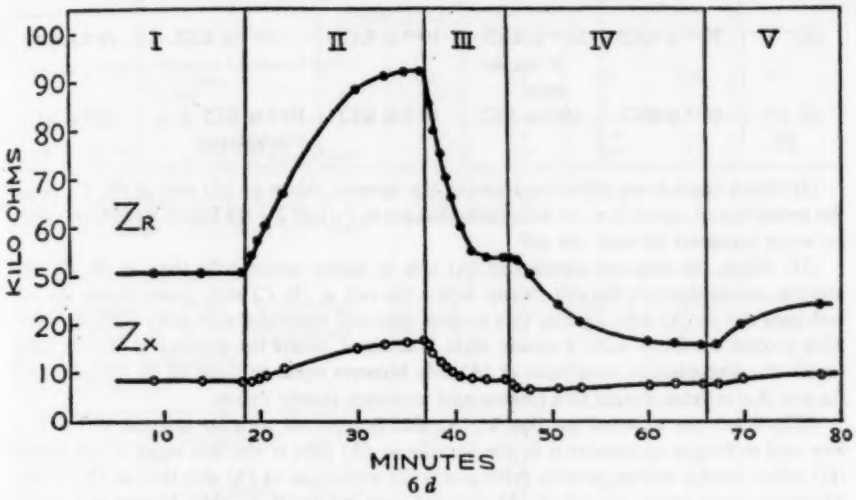
6 *b*



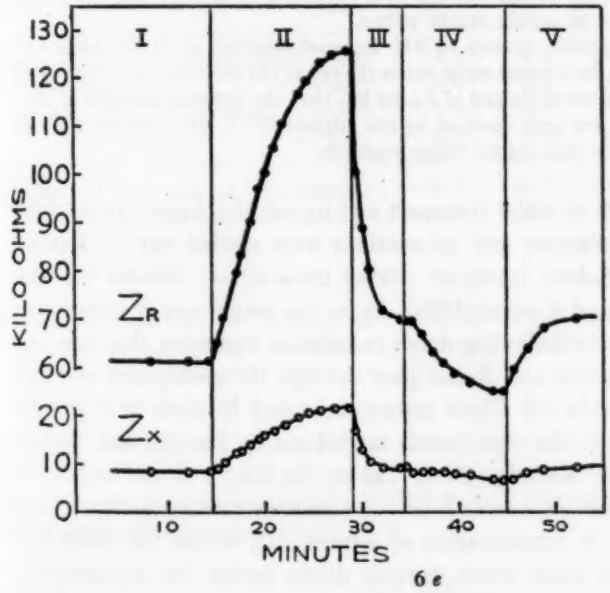
6 *c*

FIG. 6. Time courses of changes in the impedance during transcellular osmosis. In these figures real part, Z_R, and imaginary part, Z_X, of the impedance are plotted as ordinate against time in minutes as abscissa. *a*, *b*, *c*, *d*, and *e* correspond to the cases of osmotic gradients of 0.1, 0.2, 0.3, 0.4, and 0.5 mole with sucrose solution respectively. Cases (I) to (V) correspond to the processes explained in the text. No osmosis occurs in (I). Endosmosis occurs at the cell part where its impedance is measured; *i.e.*, at (B, C) side, in cases (II) and (V). Exosmosis occurs in cases (III) and (IV). At 25°C.

However, if the sucrose solution is too concentrated, harm will be done to the cell because ions or molecules inside the vacuole may be exhausted at one



6 *d*



6 *e*

half of the cell due to water transport. In such a case formation of "splits" in the chloroplast layer is actually observed.

The measurements were made by applying 10^{-2} M KCl and 10^{-2} M KCl + n M sucrose solution to the three pools, A, B, and C, in the manner shown in Table I.

TABLE I

	I	II	III	IV	V
(A)	10^{-2} M KCl	10^{-2} M KCl + nM sucrose	10^{-2} M KCl	10^{-2} M KCl	10^{-2} M KCl
(B, C)	10^{-2} M KCl	10^{-2} M KCl	10^{-2} M KCl	10^{-2} M KCl + n M sucrose	10^{-2} M KCl

(I) When there is no difference between the osmotic drives at (A) and at (B, C), across the protoplasmic membrane, no detectable changes in Z_R and Z_X are found, since there occurs no water transport through the cell.

(II) When the external solution at (A) side is higher osmotically than at (B, C) side, osmosis occurs through the cell. Water enters the cell at (B, C) side, passes inside the cell, and goes out at (A) side. During this process ions and molecules also move with the water. This process continues until a steady state is attained, where the amount of osmotic drive across the protoplasmic membrane at (A) side becomes equal to those of (B, C) side. Both Z_R and Z_X increase during this process and approach steady values.

(III) When the external solution at (A) side is replaced with its original 10^{-2} M KCl, ions and molecules accumulated in the vacuole at (A) side at the last stage of the process (II) bring about a greater osmotic drive across the membrane at (A) side than at (B, C) side. Therefore water enters the cell at (A) side and goes out at (B, C) side. During this process Z_R and Z_X decrease down to certain steady values.

(IV) This is just the opposite process to (II). External solutions at (B, C) side are replaced by sucrose solution. In this case water enters the cell at (A) side and goes out at (B, C) side. The amounts of decreases of Z_R and of Z_X are less than the increases observed in (II).

(V) External solutions are again replaced by the original 10^{-2} M KCl solution. Both Z_R and Z_X increase and regain their initial values gradually.

As for the magnitude of water transport and its velocity under transcellular osmosis, detailed experiments and calculations were carried out by Kamiya and Tazawa (1956). Solute transport during transcellular osmosis was also measured by Kamiya and Kuroda (1956). As for the magnitude of transported solutes, we can make the following rough calculation assuming that ions and molecules move with water and do not pass through the protoplasm and that sucrose does not enter the cell. These assumptions may be made as a first approximation according to the experiments carried out by Kamiya and Tazawa (1956) and Kamiya and Kuroda (1956). Taking the length of one half of the cell subjected to n molar sucrose as l_1 cm. and another half as l_2 cm., we can calculate the decrease in concentration of solutes, ΔC, within the latter half of the cell at a steady state where osmotic drives across the membrane at both sides of the cell are equal. The condition at such a steady state is expressed as follows:—

$$(0.29 - \Delta C) - 0.02 = \left(0.29 + \frac{l_2}{l_1}\Delta C\right) - (0.02 + n) \tag{16}$$

In this equation, 0.29 means the original osmotic equivalent of the cell sap, which was determined with the ordinary plasmolysis method and 0.02 corresponds to the osmotic equivalent of 10^{-2} M KCl solution. From this equation we can calculate ΔC very easily.

TABLE II

Osmotic pressure difference between A and (B, C) side, *molar*			0.1	0.2	0.3	0.4	0.5
Length of cell, mm.	At A side		11.0	8.5	19.0	15.0	17.0
	At (B, C) side (added)		15.0	16.0	24.0	14.5	17.0
Changes in concentration of cell sap at (B, C) side, ΔC, *molar*. Calculated with equation (17)			0.04	0.07	0.13	0.20	0.25
Expected changes in resistance of cell sap at (B, C) side, *per cent*, calculated with equation (18)	Endosmosis		+15.5	+31.5	+81.5	+222.0	+615.0
	Exosmosis		−12.8	−19.3	−31.5	−40.1	−46.3
Real part of the impedance, Z_R, *kilo ohms*	Endosmosis	Initial	49	40	52	50	61
		Final	52	47	74	92	125
	Exosmosis	Initial	50	42	60	54	70
		Final	49	35	45	35	55
Imaginary part of the impedance, Z_X, *kilo ohms*	Endosmosis	Initial	8.2	6.8	8.8	8.0	8.5
		Final	8.4	7.4	10.5	16.4	22.0
	Exosmosis	Initial	8.0	6.9	9.1	8.4	9.3
		Final	8.0	6.5	8.0	7.4	6.6
Changes in resistance of cell sap, $\Delta r_s/r_s$, *per cent*, calculated with equations (10) and (11)	Endosmosis		+12.5	+46.7	+81.5	+168.0	+177.5
	Exosmosis		−4.0	−35.3	−37.1	−58.7	−21.4
Changes in resistance of protoplasmic membrane, $\Delta r/r$, *per cent*, calculated with equations (14) and (15)	Endosmosis		+1.36	+4.81	+10.95	+58.4	+89.7
	Exosmosis		0.0	−3.18	−6.83	−6.67	−16.45

$$\Delta C = \frac{n}{1 + \frac{l_2}{l_1}} \tag{17}$$

Assuming that the specific resistance of cell sap (r_s) is reciprocally proportional to its concentration of ions (C), the change in resistance can be cal-

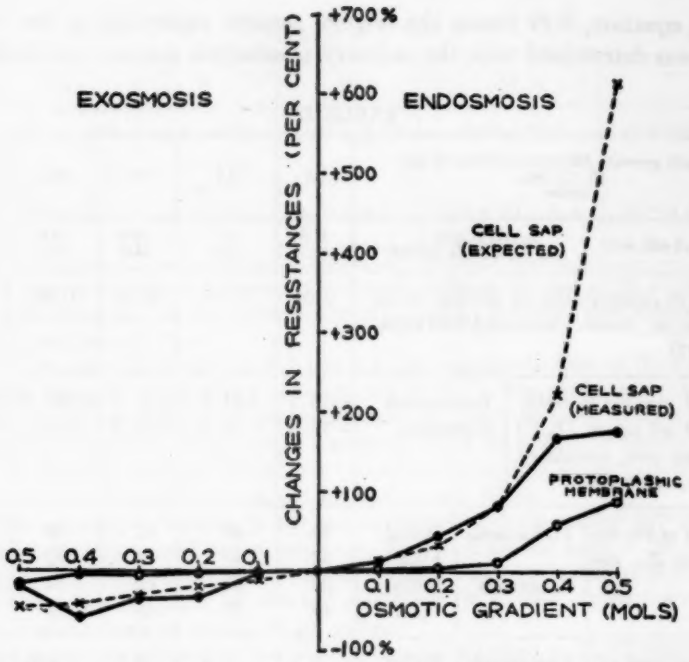


FIG. 7. Changes in resistance of cell sap and of protoplasmic membrane are plotted against osmotic gradient between A and (B, C) side. Left half corresponds to the process of exosmosis and right half corresponds to the process of endosmosis. Changes in resistance of the cell sap calculated with equations (17) and (18) are also added here. At 25°C.

culated from the change in the solute concentration of the cell sap with the following equation:

$$\frac{\Delta r_s}{r_s} = \frac{1}{1+\frac{\Delta C}{C}} - 1. \tag{18}$$

Accordingly we can compare the impedance measurements with the calculations given above at steady states. The results under transcellular osmosis with 0.1, 0.2, 0.3, 0.4, and 0.5 M sucrose are listed in Table II.

Changes in the resistance of cell sap and of protoplasmic membrane are plotted against the different concentrations of sucrose in Fig. 7, where the expected changes in resistance of cell sap calculated with equation (18) are also added. The agreement between experiments and calculations is satisfactory except in the case of 0.5 M sucrose when water goes out of the cell, and above 0.4 M when water enters the cell. Kamiya and Kuroda (1956) also found that the osmotic pressure of the vacuole of the cell does not change as much as ex-

pected from equation (17) when concentrated sucrose (0.4 or 0.5 M) is applied to the cell. When the ionic concentration of cell sap decreases greatly, ions might be freed from the protoplasm, resulting in the decrease of the electric resistance of cell sap. On the other hand, when the ionic concentration of cell sap increases greatly, ions may escape to some extent from the cell during exosomosis. In such cases the calculated changes of the ionic concentration of cell sap from equations (17) and (18) will contain some errors. Actually it was often observed that the steady state of the resistance change attained with sucrose especially at 0.4 or 0.5 M does not last long. After 30 minutes or more,

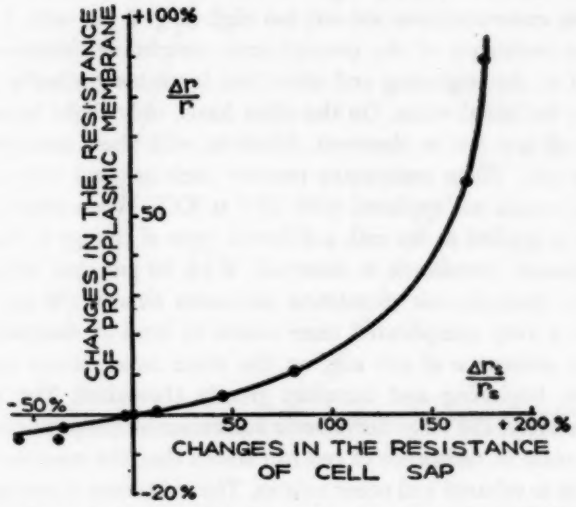


FIG. 8. Changes in resistance of the protoplasmic membrane are plotted as ordinate against changes in resistance of cell sap as abscissa. This curve is redrawn from the data in Fig. 7. At 25°C.

the increased resistances of cell sap and of protoplasmic membrane begin to change, in a pattern which is generally not simple. This is to be understood by assuming that some effects, sometimes irreversible, occurred in the cell. Anyway it can be concluded that the resistances of cell sap and of protoplasmic membrane both increase at the part of the cell where water enters, while they decrease at the part of the cell where water goes out.

Plotting the change in resistance of the protoplasmic membrane as ordinate against the change in resistance of cell sap as abscissa, we find a correlation between them as shown in Fig. 8. The resistance of the protoplasmic membrane changes almost proportionally with the resistance of cell sap up to a certain value (100 per cent increase of r_s). Beyond this, it increases more rapidly than the resistance of cell sap.

Alcohols.—According to the permeability theory of narcosis, narcotics decrease the excitability of a cell by decreasing membrane permeability. Inasmuch as impedance measurements can be used as an indication of the permeability of the protoplasmic membrane to ions, reinvestigations concerning this problem are introduced here.

The effects of ethanol are studied in this report as a representative case, because the effects of other alcohols or other fat solvents on the cell are qualitatively very similar. Changes in the resistances of the protoplasmic membrane and of cell sap under different concentrations of ethanol are shown in Fig. 9. The concentration of ethanol is expressed in volume per cent in 10^{-2} M KCl solution. When concentrations are not too high (*e. g.* 2 per cent, 4 per cent, 8 per cent), the resistance of the protoplasmic membrane decreases about 10 to 20 per cent at the beginning and after that increases gradually 20 per cent or more above its initial value. On the other hand, only slight increases in the resistance of cell sap can be observed. Alcohols, with these concentrations, do not harm the cell. These resistances recover their original values gradually, when external media are replaced with 10^{-2} M KCl. When ethanol of higher concentration is applied to the cell, a different type of change in the resistance of the protoplasmic membrane is observed. With 10 per cent ethanol the resistance of the protoplasmic membrane decreases almost 100 per cent, irreversibly, with a very complicated time course (a kind of damped oscillation (Fig. 9)). The resistance of cell sap, on the other hand, shows only a slight increase at the beginning and increases greatly thereafter. The last step of this process, namely, the large decrease in resistance of the protoplasmic membrane and increase in resistance of cell sap shows that the membrane becomes fully permeable to ethanol and other solutes. The resistance of cell sap increases gradually to the value (*i. e.* several hundred kilo ohms or more) which is presumably attained when ethanol and cell sap are freely mixed with each other through the protoplasmic membrane. Protoplasmic streaming ceases and the turgor of the cell is lost completely, indicating the death of the cell. When the cell is subjected to more concentrated ethanol, for example 20 per cent or more, oscillatory changes in resistance of the protoplasmic membrane are no longer detected clearly. They are completely masked by its rapid irreversible decrease. This process, however, does not differ essentially from the case of 10 per cent ethanol except that the time course is rapid.

When ethanol is applied to a half of the cell, some different types of changes in these resistances are observed. As an ethanol solution of a few per cent is osmotically higher than 10^{-2} M KCl, it is to be expected that water enters the cell at 10^{-2} M KCl side and goes out of the cell at the ethanol side as was described in the paragraph on osmosis. The changes in resistance of the protoplasmic membrane and of cell sap at the ethanol side are shown in Fig. 10. In the case of 4 per cent ethanol the resistance of the protoplasmic membrane

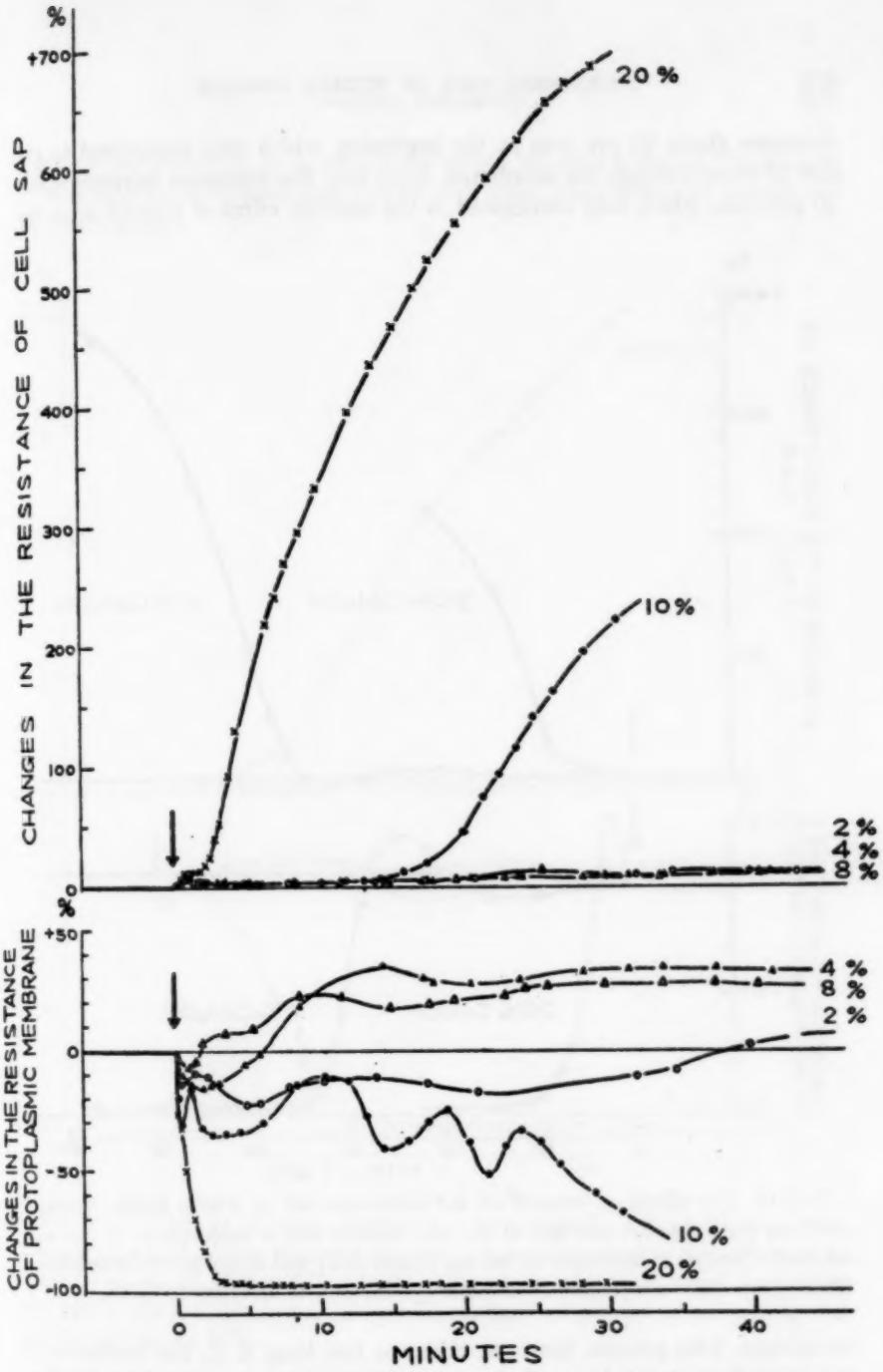


FIG. 9. The effects of ethanol on the internodal cell of *Nitella flexilis*. Ethanol solutions were added to the whole of the cell. Concentrations are expressed with volume per cent of ethanol in 10^{-2} M KCl solution. Changes in resistance of cell sap (upper half) and of the protoplasmic membrane (lower half) are plotted as ordinate against time in minutes. At 15°C.

decreases about 10 per cent at the beginning, which may correspond to the exit of water through the membrane. After this, the resistance increases about 20 per cent, which may correspond to the narcotic effect of ethanol upon the

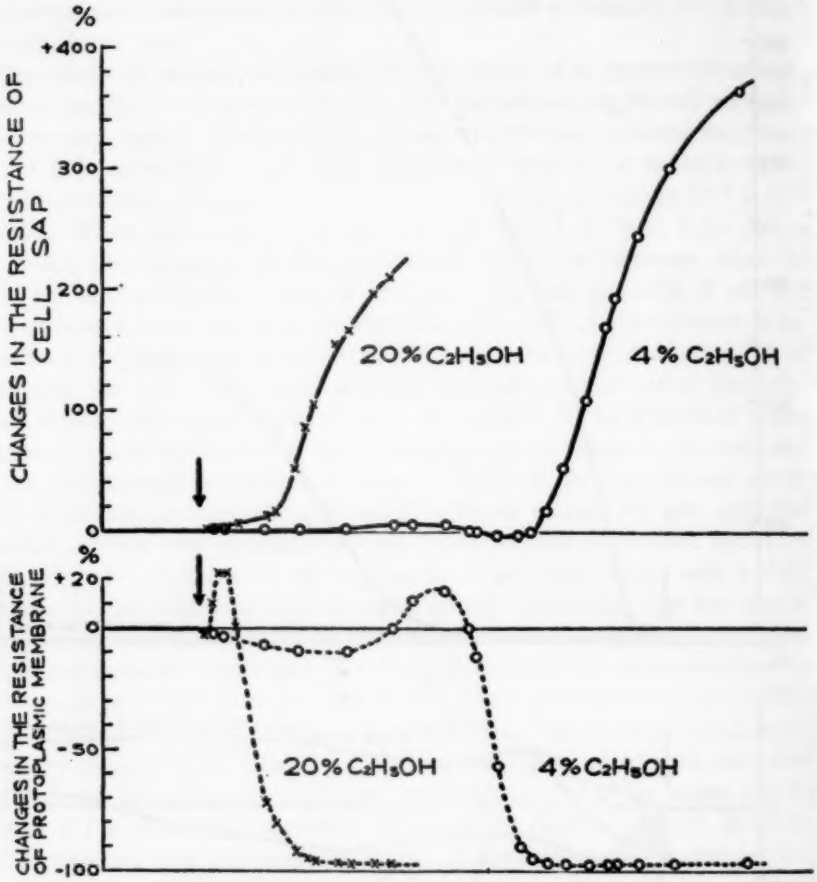


FIG. 10. The effects of ethanol on the internodal cell of *Nitella flexilis*. Ethanol solutions were added to one half of the cell. Another half is subjected to 10^{-2} M KCl solution. Changes in resistance of cell sap (upper half) and of the protoplasmic membrane (lower half) are plotted as ordinate against time in minutes. At 25°C.

membrane. This process, however, does not last long; *i. e.*, the resistance of the protoplasmic membrane decreases rapidly and irreversibly about 100 per cent. At this stage ethanol is considered to have entered the cell and to have injured it. The resistance of the cell sap, on the other hand, increases slightly at the beginning and greatly later after a short period of decrease. With much

higher concentrations (*i. e.* 20 per cent or more), the time course of the changes in these resistances is much faster than with lower concentrations, though the process is similar.

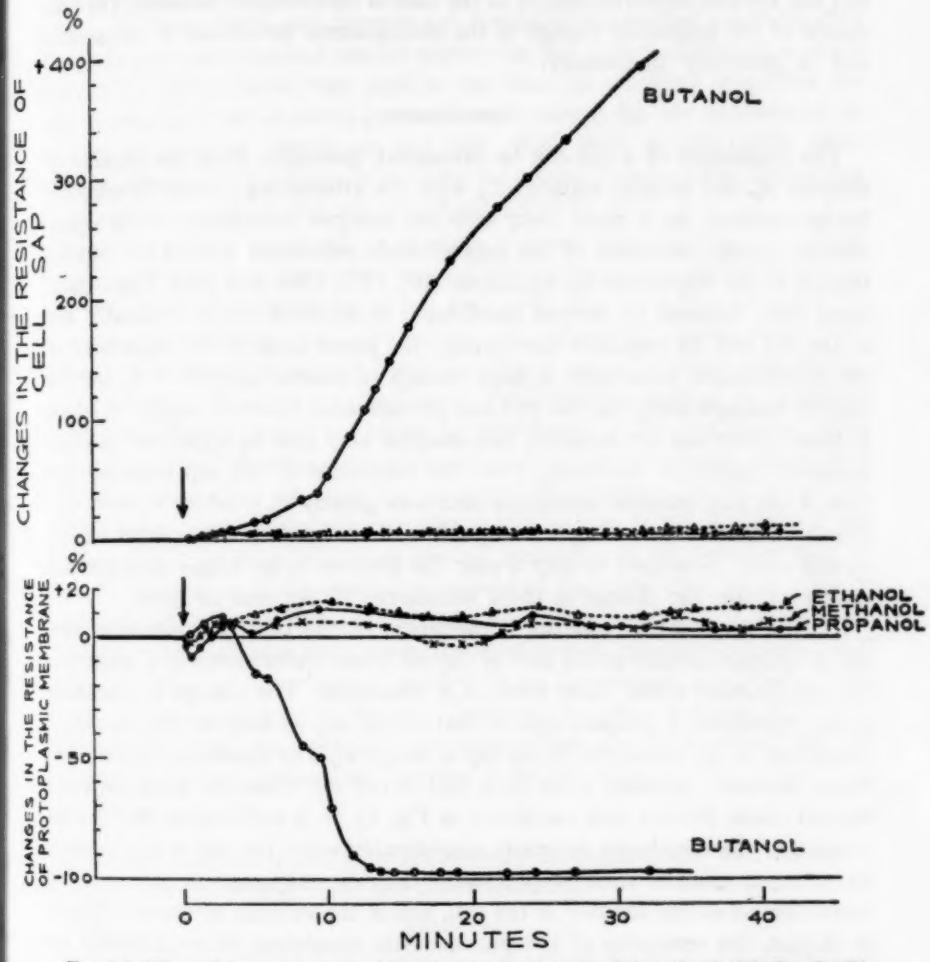


FIG. 11. The effects of some alcohols on the internodal cell of *Nitella flexilis*. These were added to the whole of the cell. Their concentrations are expressed as volume per cent in 10^{-2} M KCl solution. Changes in resistance of cell sap (upper half) and of the protoplasmic membrane (lower half) are plotted as ordinate against time in minutes. At 16°C.

The effects of homologous series of alcohol, *i. e.* methanol, ethanol, propanol, and butanol, on the cell are compared in Fig. 11. These are all applied to the whole of the cell at a concentration of 4 per cent. Methanol, ethanol, and propanol increase the resistance of cell sap only slightly and increase the resist-

ance of the protoplasmic membrane about 10 per cent. Butanol, however, harms the cell at the same concentration. Irreversible decrease of the resistance of the protoplasmic membrane and irreversible increase of the resistance of the cell sap are also observed here as in the case of concentrated ethanol. The time course of the resistance change of the protoplasmic membrane is not smooth but is generally undulatory.

DISCUSSION

The impedance of a cell can be calculated, generally, from the parallel resistance R_p and parallel capacity C_p with the alternating current Wheatstone bridge method. As is made clear with the analysis introduced in the report, changes in the resistance of the protoplasmic membrane and of cell sap are related to the impedance by equations (10), (11), (14), and (15). These equations were deduced on several conditions: in addition to the favorable size of the cell and its negligible size change, the phase angle of the impedance of the protoplasmic membrane is large enough to assume $(\omega c_3 r_3)^2 \ll 1$, and the electric leakages along the cell wall and protoplasmic layer are negligibly small. If these conditions are satisfied, this analysis may also be applicable to other biological materials. However, when the resistance of cell sap increases and that of the protoplasmic membrane decreases greatly (*i. e.* under harmful conditions), the electric leakages along cell wall and protoplasmic layer cannot be neglected. Therefore, in such a case the analysis is no longer accurate but underestimates the change in these resistances 10 per cent or more.

Under "transcellular osmosis," resistances of the protoplasmic membrane and of cell sap increase at the part of the cell where water enters (*i. e.* endosmosis) and decrease where water exists (*i. e.* exosmosis). The change in resistance of the membrane is proportional to that of cell sap as long as the amount of the change in the resistance of cell sap is not great. The resistance of the membrane, however, increases more than that of cell sap when the latter increases beyond about 100 per cent (as shown in Fig. 8). It is well known that the resistance of the membrane decreases considerably when the cell is in a concentrated ionic solution such as potassium chloride. Although in our case the concentration of the interior of the cell, not of the exterior solution, is forced to change, the resistance of the protoplasmic membrane shows a similar response to change in concentration of the solution in contact with it. In the case of electric current flow across the protoplasm, the resistance of the squid axon membrane increases under the anode, and decreases under the cathode where cations such as potassium ions are considered to accumulate (Cole and Baker, 1941). The situation is very similar to our case except for the difference in the flowing medium (namely, water or electric current). Referring to these results, the greater part of the resistance change of the membrane under transcellular osmosis may probably be brought about by the change in the ionic concentration of cell sap.

Alcohols are well known as narcotics for nerve and other cells. According to the permeability theory of narcosis, narcotics suppress the excitability of a cell by decreasing ionic permeability or preventing its increase (Heilbrunn, 1952; Johnson, Eyring, and Polissar, 1954). Bozler and Cole (1935), Dubuisson (1937), Guttman (1939), and Fessard and Kohler (1951) studied the impedance of muscle cells and obtained results supporting this theory. Although a single plant cell, *Nitella flexilis*, was used in our case, its electrical properties are very similar to those of nerve or muscle cells except for the slowness of its response to stimulus (Osterhout and Hill, 1930, 1935; Blinks, 1930, 1936; Cole and Curtis, 1938). Alcohols or other fat solvents prevent the conduction of the action current of this cell (Osterhout and Hill, 1930).

When the cell is subjected to alcohols of lower concentration, an initial decrease and later increase in resistance of the protoplasmic membrane can be observed. Such phenomena occur not only with alcohols but with other fat solvents, *i. e.* acetone, ether, chloroform, etc. Numerous investigators have shown a preliminary increase in the irritability of nerve or muscle cells following treatment with narcotics (Heilbrunn, 1952). Initial decrease in resistance of the protoplasmic membrane seems to correspond to this stimulating effect of narcotics. The following increase in resistance of the membrane corresponds to the narcotic effect of these fat solvents. Non-propagation of the action current under the influence of narcotics might be due to the inability of the protoplasmic membrane to induce enough excitation at the adjacent part of the cell, on account of the resistance increase of the membrane, or to a rise of the threshold value at that part of the cell.

Rapid and remarkable decrease in the resistance of protoplasmic membrane under the effect of concentrated alcohols is an irreversible one, which corresponds to the injury done to the cell. Therefore this process should be strictly distinguished from the reversible processes which are detected as the preliminary decrease or subsequent increase in the resistance of the protoplasmic membrane.

The author desires sincerely to acknowledge his indebtedness to Professor N. Kamiya for his valuable criticism and encouragement.

SUMMARY

Impedance changes of single plant cells of *Nitella flexilis* were studied under different environmental conditions.

With the analysis presented changes in resistance of the protoplasmic membrane and of cell sap can be studied independently and simultaneously.

Under "transcellular osmosis," the resistance of the protoplasmic membrane and of the cell sap increase at the part of the cell where water enters, while they decrease where water goes out.

Ethanol of low concentration (below 8 per cent) first decreases and later increases the resistance of the protoplasmic membrane. Concentrated ethanol

(over 10 per cent), however, brings about a large decrease in resistance of the protoplasmic membrane. Its time course is not simple, but undulatory changes occur.

When ethanol is applied to one part of the cell, the resistance of the protoplasmic membrane shows a different type of change, which may be attributed to the local osmotic effect of ethanol; injury generally occurs with comparatively low concentration.

Methanol, ethanol, and propanol have almost the same effect upon the cell, while butanol is toxic at the same concentration.

When the cell dies, the resistance of the protoplasmic membrane decreases greatly, while the resistance of the cell sap increases to a level (several hundred kilo ohms or more), expected when external solution and cell sap are freely mixed with each other.

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MAINTAINED ACTIVITY IN THE CAT'S RETINA IN LIGHT AND DARKNESS*

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(Received for publication, November 5, 1956)

INTRODUCTION

Maintained activity in the absence of obvious external stimuli has been observed in several sense organs of vertebrates and of invertebrates (Tasaki and Davis, 1955; Katsuki, Yoshino, and Chen, 1950; Bullock and Diecke, 1956; Cohen, 1955; Adrian, 1937; Löwenstein and Sand, 1940; Zotterman, 1953; Roeder, 1955; Autrum, 1952; see Granit's review, 1955). Such "spontaneous" discharges are of special interest in the retina because of its similarity in neuronal organization to the higher nervous centers in which continuous activity is well known (Bremer, 1949). The presence of maintained activity is also related to the problem of transmission of sensory information. It implies that visual stimuli are transmitted by modulation of ever present background discharges. The occurrence of a background discharge with a random component, or "noise," is essential evidence in support of the view that sensory thresholds should be regarded as signal/noise discrimination problems (Hecht, 1945; Tanner and Swets, 1954; Gregory and Cane, 1955; FitzHugh, 1957, Barlow, 1956), and it was therefore important to make sure that such noise was constantly present in the best preparations, and was not the product of experimental interference. In this paper the statistical properties of the maintained discharge are analyzed, and the changes in maintained impulse frequency with different levels of steady illumination and in the absence of light are described.

The cat's eye is well suited for these studies since it can be used unopened without appreciable surgical interference and with its circulation and optical pathways intact. Good physiological conditions are especially important in the registration of spontaneous discharges which are very sensitive to environmental changes. The great increase of sensitivity during dark adaptation gives an indication of the good condition of the present preparation. Further, the eye is isolated from possible efferent centrifugal nervous control and light can be excluded, thus providing a sense organ cut off from external stimuli.

* Supported by a research grant from the National Institutes of Health, United States Public Health Service.

The subsequent papers (Barlow, FitzHugh, and Kuffler, 1957 *a*, *b*) will deal mainly with changes of organization of the receptive field during dark adaptation and with the measurement of absolute threshold for the discharge of a single ganglion cell. The last paper will present a statistical analysis of threshold responses to short flashes (FitzHugh, 1957).

METHOD

Preparation

Unless otherwise noted, adult cats were decerebrated under ether or surital (thiamylal sodium, a barbiturate of short action) by opening the skull and cutting across the mesencephalon just anterior to the tentorium. After removal of the cerebrum, cranial nerves III, IV, V, and VI were crushed or cut intracranially to prevent movements of eye and facial muscles. In some decerebrate preparations subparalytic or paralytic doses of tubocurarine were used. Many preparations stayed in good condition for 8 to 15 hours.

The preparation was set up for retinal recording by the technique of Talbot and Kuffler (1952); the eye was closely fixed to a ring by the conjunctiva, a hypodermic needle pushed through the sclera just behind the ciliary body, and an electrode inserted through the needle until it touched the retina, as observed through the magnifying eyepiece of the multibeam ophthalmoscope (see below, under Equipment).

Experimental observations were started more than 3 hours after the cat came off ether, so that in this case residual anesthetic was unimportant. The same is probably true after surital, since a test showed that a cat was fully awake, but its muscular coordination was not quite normal, in 3 to 4 hours. Progressive physiological changes attributable to initial anesthesia were never noticed.

Equipment

The optical equipment and general experimental set up have already been described in detail (Talbot and Kuffler, 1952). The main instrument, a multibeam ophthalmoscope, had a base with a headholder for rigid fixation of the preparation. The eye was held fixed to a ring which also carried the microelectrode holder and electrode-advancing mechanism. Above the head was the optical viewing and stimulating system. This was aligned with the eye and could be freely tilted and rotated, to direct light beams to various retinal regions. The eye was thus in the center of the spherical coordinate system of the movable optical system. A tungsten filament lamp served for background illumination for light adaptation and at the same time provided light for viewing of the cat's fundus through a microscope eyepiece. The background light covered a roughly circular area 16 to 19° in diameter (equivalent to 3.7 to 4.5 mm. on the cat's retina). The intensity of this background was changed both with neutral filters and by altering the current flowing through the lamp, and its color was controlled by using Wratten filters. A second light source was a glow modulator tube (Sylvania R1131C) driven from an electronic stimulator. This gave square pulses of light of constant intensity and variable duration. This light passed through two Eastman circular neutral wedges giving an optical density variable through 7 log units, and, if needed, a Wratten color filter. A diaphragm was placed

in a position parfocal with the retina and could be revolved to give a series of concentric spots ranging in diameter from 14°3 minutes to 0°36 minutes (equivalent to 3.25 to 0.135 mm. on the cat's retina). Annular patterns with inside diameters ranging from 0°50 minutes to 3°30 minutes (0.80 to 1.90 mm. on the retina) were also available. The procedure for measuring the illumination of the retina in light and energy units from these two beams will be described in a following paper (Barlow, FitzHugh, and Kuffler, 1957 *a*).

Light from the stimulator passed through a contact lens on the cat's cornea and the natural pupil which was dilated by section of nerve III. Since the contact lens was not suited to every cat, an additional spectacle lens was frequently needed to focus accurately, but as it was of low power and close to the eye, it did not change the magnification appreciably.

Nerve or ganglion cell potentials were recorded by electrodes of three types: glass-insulated platinum-iridium wires of 10 to 15 μ in diameter of the type used by Granit (1947), by indium-filled, gold- and platinum-plated, glass capillaries with a tip to 3 to 6 μ as recently described by Dowben and Rose (1953), and by micropipettes of less than 1 μ tip diameter, filled with 3 M KCl. The electrodes were advanced by a fine screw or by a hydraulic mechanism remotely controlled by a micrometer screw. The electrode holder could be moved on a ball-and-socket joint so as to reach different points on the retinal surface. At each position, the electrode was slowly advanced so as to touch or penetrate the retina. The platinum electrodes were allowed merely to touch the retina lightly, while the indium and micropipette electrodes penetrated the internal limiting membrane. Suitable single units (ganglion cells) were usually found only after trying a number of positions on the retina when platinum or indium electrodes were used, giving potentials of 100 to 500 microvolts. With micropipettes the potentials were sometimes many millivolts and were obtained at practically any point on the retina. The usual criteria for a single unit discharge were used: uniformity of size, shape, and sound of the action potentials. Multiunit discharges were not used. The stimulus spot was centered on the tip of the electrode above the ganglion cell, which lies in the center of the receptive field, and the response to a flash there indicated whether the cell was an on- or off-center unit. The metal electrodes were connected to a conventional physiological amplifier, cathode ray oscilloscope, and loud-speaker. The 3 M KCl-filled capillaries were connected through a negative capacity feed back input stage (MacNichol and Wagner, 1954) to a direct coupled amplifier.

Counting of Nerve Impulses

The nerve impulse signal from the amplifier was used to trigger an electronic counter which periodically counted the number of impulses occurring during a 10 second period, three times a minute. If the action potentials were large relative to the noise level in the baseline, the triggering voltage of the counter could easily be adjusted so that all the impulses but none of the baseline variations were counted. However, if the action potentials were relatively small, the variation in their heights due to the noise could cause some of the impulses to be missed by the counter. In the earlier experiments, a check of counting accuracy was made periodically by displaying the output pulse from the counter on one beam of the cathode ray oscilloscope,

the action potential on the other, and seeing if any missed impulses could be detected with a fast repetitive sweep. In the later experiments, a device to brighten the cathode ray oscilloscope trace of the impulse at the instant of triggering served the same purpose.

RESULTS

A. General Properties of the Maintained Discharge

Maintained activity in single ganglion cells of the cat has frequently been observed by Granit (1947) and Noell (1953), and has been regularly seen by Kuffler (1952; 1953). Experiments, however, were needed to establish specifically the physiological nature of the background discharge. Ganglion cell activity was influenced, for instance, by electrode pressure and eye movement, poor circulation, distortion and manipulation of the eyeball, anesthetics, and changes in ventilation and temperature.

Pressure.—The most important source of artefact in the present preparation was pressure on the ganglion cells. The platinum wire electrode, to record best, must lie over and quite near the ganglion cells, and around its area of contact it always presses to some extent on the cells. Once a ganglion cell was located, a constant light flash caused a reasonably reproducible response. In platinum electrodes the fine wires are surrounded by a relatively thick glass jacket and appreciable pressure can be exerted without piercing the retina. If the electrode was advanced and obvious pressure exerted (detected by dimpling of the retinal surface), the following sequence of changes was frequently seen. The background activity rate was increased and usually the frequency waxed and waned. If the pressure was great enough the discharge attained a rate of several hundred per second and was followed by an abrupt cessation of activity, obviously due to injury. This high pitched injury discharge of an agonized cell, familiar to most investigators, is readily recognized in the loudspeaker. Pressure with the electrode, if carefully and gradually applied, may lead to a reversible block of the spike in the ganglion cell soma, leaving behind a local potential (*cf.* Fig. 2, Kuffler, 1953).

In many ganglion cells advancing the electrode did not cause the changes mentioned above, although obvious pressure was exerted. It is assumed that in such instances the electrode was not located directly above the active cell. Iridium electrodes (see Method) with an outer tip diameter of 3 to 4 μ, or the smaller fluid electrodes, penetrated the different retinal layers without difficulty. Ganglion cell discharges appeared at definite depths of the advancing electrode. Activity in individual cells was often readily and repeatedly obtained at a definite depth during fine up-and-down adjustments. Under these conditions the electrode clearly did not injure the cell since the average maintained discharge frequency did not change.

Another way of studying maintained background discharge is by recording

from optic nerve fibers. In such a case, electrode pressure cannot affect the activity, which originates in a receptive field some distance from the electrode. The same is true when an electrode touches lightly the surface of the retina at any place (except in the area centralis, where there are few nerve fibers—unpublished data). The triphasic axon discharges recorded simultaneously from

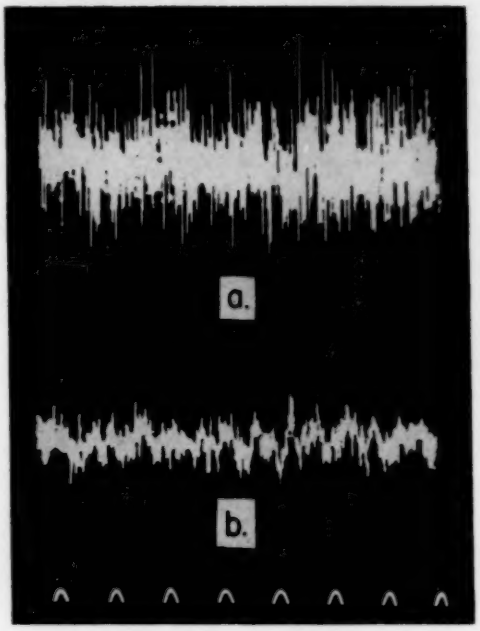

FIG. 1. Potentials recorded from the unopened eye of the cat with a platinum electrode and with no retinal illumination. (*a*) Massed spontaneous discharge from nerve fibres, electrode touching the surface of the retina. (*b*) Noise level with electrode tip withdrawn into the vitreous. Time signal 10 msec. The largest action potentials in (*a*) have been retouched; the smaller ones are not seen individually but rather as a broadening of the baseline as compared with (*b*).

many fibers are readily distinguished from those of single ganglion cells. It is impossible with present methods to record for prolonged periods from individual nerve fibers and to estimate whether units are active continuously or perhaps in relays only. Nerve fiber records (Fig. 1), however, clearly show, in agreement with records from single ganglion cells, that massive continuous nervous activity is a feature of the light- or dark-adapted retina. The activity in Fig. 1 *a* is affected by illumination of the eye while the record in Fig. 1 *b* is not changed. The increased baseline disturbance on touching the retina is therefore not due to an increased input resistance.

After a single ganglion cell was obtained, the discharge rate and rhythm sometimes changed suddenly, as a result of movement of the eyeball, due to coughing, jerking, etc. On occasions the discharge followed the rhythm of the heart beat. All these changes were due to pressure effects and could be avoided by different placement of the electrode, better fixation of the eye or, in a few instances, by curarization.

Circulation, Respiration, Temperature.—In our preparation the circulation of the retina can be examined during the experiment. Deterioration in the cat's condition could frequently be detected in this way prior to the onset of more obvious general symptoms. Slowed movement of red cells in the capillaries and finer vessels was sometimes seen, frequently accompanied by "clumping and sludging," a phenomenon also seen in other organs when they were poorly circulated; *e.g.*, in the surface vessels of muscles. In some experiments the circulation to the eye was purposely cut off for varying periods by clamping the carotids. This was promptly followed by a decrease of volume of the retina resulting in a slight withdrawal of the retina from the electrode. By advancing the electrode, contact could be reestablished and frequently ganglion cell discharges continued for several minutes before becoming irregular and dropping out. No serious attempt was made to study in detail the effect of poor circulation on retinal activity, but the impression was formed that units can recover well after temporary arrest of blood flow. As a result of impaired circulation the general level of activity, *i.e.* the number of active units as estimated by nerve fiber potentials, seemed to be decreased and in some units the background discharge eventually slowed or stopped altogether. In the eyes of decerebrate cats which had apparently good circulation, all ganglion cells showed maintained activity after a period in darkness or during steady illumination (see later).

Some cats were hyperventilated by a respirator or were made to breathe into a balloon. Again, these experiments were carried only so far as to make reasonably certain that these factors were not responsible for or did not greatly influence maintained activity as studied in these investigations. The same was true for cooling of the eyeball by cold saline and letting the body temperature fall to about 34°C. from the usual 37–38°C.

Anesthetics.—The most convenient way of experimenting on the eye is during deep anesthesia; dial-urethane or pentobarbital sodium (nembutal) was frequently used. Some features of the retinal discharge were, however, affected by anesthetics. The effect of nembutal on retinal discharges was tested by injecting it intravenously into decerebrate preparations while recording from a ganglion cell. The action of a dose, about one-fourth that needed for general anesthesia, may be summarized as follows. (1) The maintained activity was decreased or suppressed but generally returned to its original level within a few minutes. When it was reestablished, however, it often seemed to have different characteristics from the normal maintained discharge. Sometimes the

discharge became less regular, but it is also noteworthy that the most regular discharge we ever recorded was from an anesthetized preparation. Perhaps the most constant finding was that impulses tended to be grouped and various rhythms appeared; it would demand an elaborate statistical analysis to describe these completely. (2) The response to a light flash of 0.5 to 1.0 seconds, which in the absence of the drug did not outlast the stimulus, became prolonged and increased in frequency. For instance, an on-response might be followed by an after-discharge lasting 5 seconds. On occasions the discharge was then suddenly cut off.

When dial-urethane or nembutal was used alone, without decerebration, their specific effects were not easy to assess. It seemed that some features of the responses during prolonged flashes, of 1 to 10 seconds or longer, were changed. In decerebrate cats, maintained illumination of a small central area in an on-center unit produced an increased discharge rate for many seconds or minutes rather than the shorter transient on-responses generally seen in anesthetized animals. Further, the maintained on-discharge could also be obtained more readily by stimulating the surround of an off-center unit with an annular pattern of light that did not fall on the center of the receptive field.

The present experiments lead to the definite conclusion that all retinal units which are accessible by the present recording methods are in continuous irregular activity when dark-adapted and when light-adapted.

B. Changes in Discharge Rates at Different Levels of Illumination

After a single unit was found, only the background light, covering the entire receptive field, was altered. The maintained activity was present in all units whether in light or dark-adapted and was measured by counting impulses, either during ten 1 second periods per minute or during three 10 second periods per minute. Both procedures gave similar results.

This method of counting impulses did not resolve any transient changes of frequency that occurred within several seconds following a sudden change of illumination. The cell population was selected without regard to type (on- or off-center), generally from the upper half of the retina in the region of the tapetum. No distinctive pattern of maintained discharge behavior has emerged but some representative samples will be described. Most of our observations were started at a light-adapted level, and we tried to record from a unit for about 30 minutes before the background illumination was changed, or until at least a steady initial baseline of frequency was reached. Those units which gave irregular discharges with wide fluctuations of frequency were arbitrarily discarded because in many instances the changes in discharge rates were accompanied by some detectable movements of the eye. After a change in background illumination there was generally a large transient change in frequency, lasting between 5 and 15 minutes, followed by a fairly constant frequency level.

Fig. 2 illustrates an off-center unit which discharged at about 15/second for

30 minutes while under 3.0 f.-c. retinal illumination, recorded with a platinum electrode. In this and the following figures, there were too many readings to be plotted as separate points. Instead, the solid line was drawn through the points to indicate mean frequency; the dotted lines above and below enclose all but a few of the readings. When the illumination was reduced to 0.03 f.-c. there occurred a sudden high frequency burst of activity (not plotted) following which the frequency returned to 80 to 90/second within a minute. After another 15 to 20 minutes the discharge frequency levelled off to a rate near 55/second

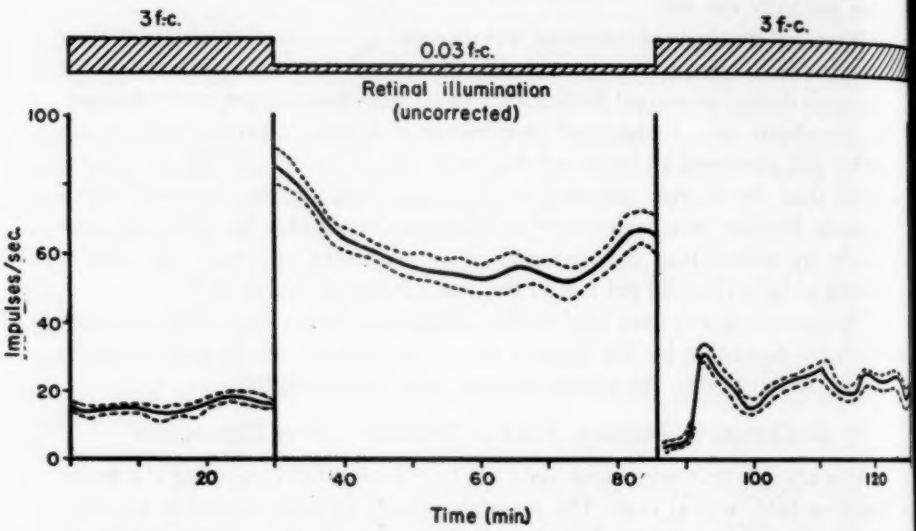

FIG. 2. Frequency of maintained discharge during changes of retinal illumination. Off-center unit recorded with platinum electrode. Values of retinal illumination are uncorrected for all losses in the ocular media. Solid line indicates mean frequency; dotted lines are limits of variation.

which was maintained for more than half an hour. The background illumination was then increased once more to 3.0 f.-c. This time the discharge disappeared briefly and then returned, during 10 to 15 minutes, to a maintained level near 20/second which was counted for another 30 minutes until the unit was lost.

While in the previous experiment the discharge rate increased as the illumination was reduced, the opposite result is shown in Fig. 3. This ganglion cell, an on-center unit, had a low discharge rate of 4 to 8/second for a 40 minute period at an illumination of 0.3 f.-c. A tenfold increase of illumination to 3.0 f.-c. increased the discharge rate to 14 to 17/second, which remained constant for 35 minutes. It should be noted that there was no marked transient discharge shift during the first 5 to 15 minutes after increasing the illumination; the new maintained rate was established practically at once. This unit was recorded with a KCl-filled micropipette of about 0.5 μ tip diameter.

A third example is plotted in Fig. 4, an on-center unit which was recorded for 3 hours with a KCl-filled electrode. Observations were started with the

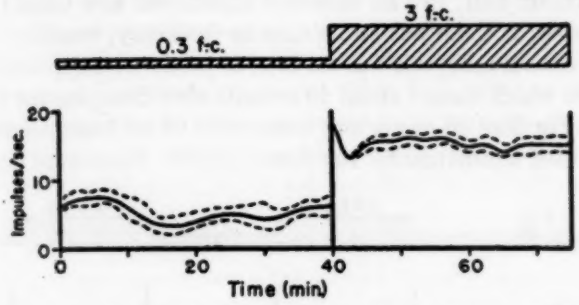

FIG. 3. Frequency of maintained discharge during a change of illumination. On-center unit recorded with micropipette. New maintained discharge rate at 3.0 f.-c. established almost immediately, in contrast to Fig. 2.

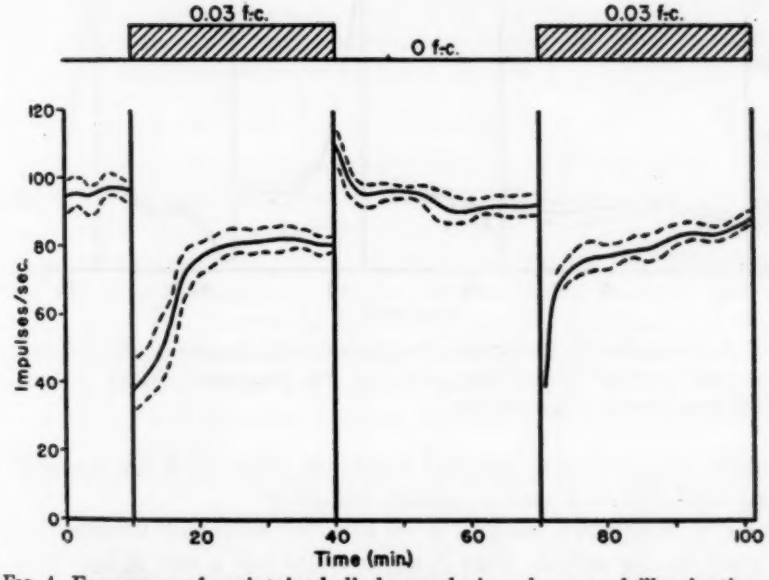

FIG. 4. Frequency of maintained discharge during changes of illumination. On-center unit recorded with micropipette. Maintained frequency decreased with increased illumination, in contrast to Fig. 3.

retina in total darkness and a discharge rate of 92 to 100/second. Following illumination of 0.03 f.-c. the first recorded frequency was decreased and it was 10 to 15 minutes before a new steady level of 80 to 85/second was established. After 30 minutes the light was turned off again, resulting in a transient increase of frequency lasting 5 minutes, followed by a steady rate of 90 to 95/second.

After a return to the 0.03 f.-c. background, the previous pattern was repeated. It should be pointed out that from Fig. 3 the opposite result might be expected from an on-center unit; *i.e.*, an increased maintained rate when the illumination is increased. If there was an increase in frequency, resulting from stimulation of the central zone, this was too brief to produce an appreciable effect on the first count which started about 10 seconds after changing the illumination.

Finally, in Fig. 5 an off-center unit is shown in which backgrounds of 3.0 and 24 f.-c. were used alternately for 10 minute periods. There were large transient

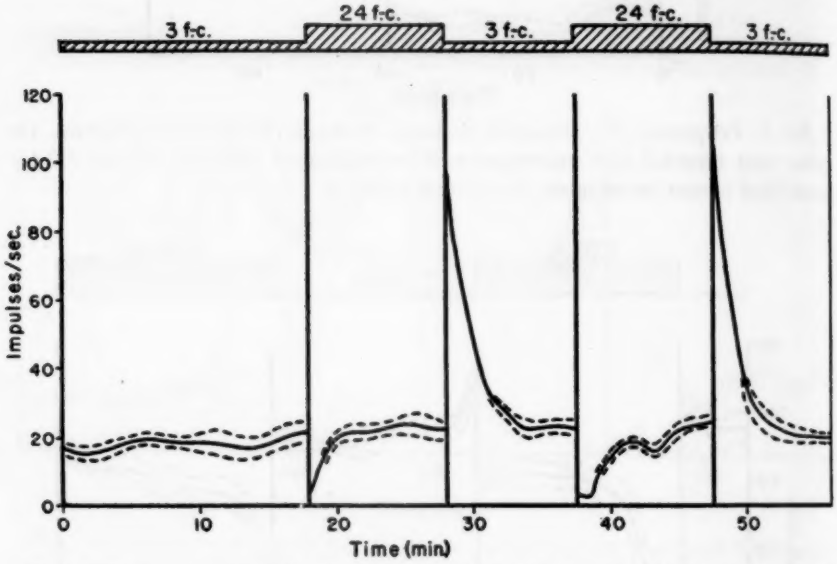

FIG. 5. Frequency of maintained discharge during changes of illumination. Off-center unit recorded with indium electrode. No permanent change of frequency resulted from change of illumination.

frequency changes lasting less than 5 minutes, after which the frequency returned each time to a nearly constant frequency.

In a few experiments, portions of the receptive fields were illuminated separately for varying periods. Thus in an on-center unit a spot of light of about 0.5 mm. in diameter increased the maintained discharge rate. Illumination of the surround of the same field by a ring of light which did not shine on the central region suppressed or decreased the discharge. When the whole receptive field was illuminated (as in Figs. 2 to 5) the discharge rate was intermediate between the activity produced by exclusive excitation of the center or of the surround. These results suggested that the discharge evoked by light, though it adapted rapidly to begin with, did not adapt completely. The maintained discharge could then be explained as the residue of the light-evoked discharge,

and its net amount should be the result of the interaction of the non-adapting residue of the excitatory and inhibitory influences converging on the ganglion cell. Although this is a relatively simple hypothesis it would not work out so simply in practice because the receptive field organization itself changes when the level of illumination is changed (Barlow, FitzHugh, and Kuffler, 1957 *b*). Thus an increase of illumination over the whole field might cause an increase or a decrease of frequency depending on whether the on-region or the off-region of the receptive field was dominant, or there might be no change if they were evenly balanced. Furthermore, which was dominant would vary, not only from ganglion cell to ganglion cell, but also with the level of the adapting light and the past history of the cell. The results of Figs. 2 to 5 do not, therefore, prove or disprove the hypothesis: to settle the matter it would be necessary to follow the change in receptive field organization together with the change in level of maintained activity, and this has not been done.

It might be thought that the level of maintained activity would change during the course of dark adaptation. As described elsewhere, many units were successfully held throughout a period of dark adaptation, but no correlation between dark adaptation levels and discharge frequencies was noticed, apart from the transient changes in the first few minutes.

C. Statistical Properties

There were four steps in the statistical analysis of the apparently random discharge. (*a*) The intervals were measured and arranged in histograms. (*b*) The hypothesis that the probability of occurrence of an impulse is constant and does not vary with the time of the preceding impulse was tested; the hypothesis leads to the expectation of an exponential distribution of intervals, and this was shown not to fit the data. (*c*) The gamma distribution was shown to fit the data adequately. (*d*) By using serial correlation coefficients it was shown that the impulses do not occur at random from this distribution, but there is a slight but significant tendency for short intervals to be followed by long ones and *vice versa*.

Fig. 6 shows a typical maintained discharge from a single off-center ganglion cell; the intervals between successive impulses vary in an apparently random fashion over a wide range. Such a record resembles at first sight a discharge of pulses from a Geiger counter near a radioactive source; in the latter case the different pulses are statistically independent events, except that the counter has a brief "dead time," following each pulse, during which it is unresponsive. The distribution curve of intervals (t) in a Geiger counter discharge with dead time d is closely approximated by an exponential function (Alaoglu and Smith, 1938):

$$\begin{aligned} f(t) &= 0 && 0 \leqq t < d \\ &= me^{-m(t-d)}, && d \leqq t < \infty \end{aligned} \tag{1}$$

In order to compare the ganglion cell discharge statistically with that from a Geiger counter, the values of the intervals were measured from records of the maintained discharge from six different units from three cats. The number of intervals measured for each unit was between 144 and 400. First, histograms were plotted to show the experimental distribution of interval durations. Figs. 7 *a* and *b* show two such histograms, labelled "experimental." In an attempt to fit formula (1) to the histograms, the parameter d was chosen as

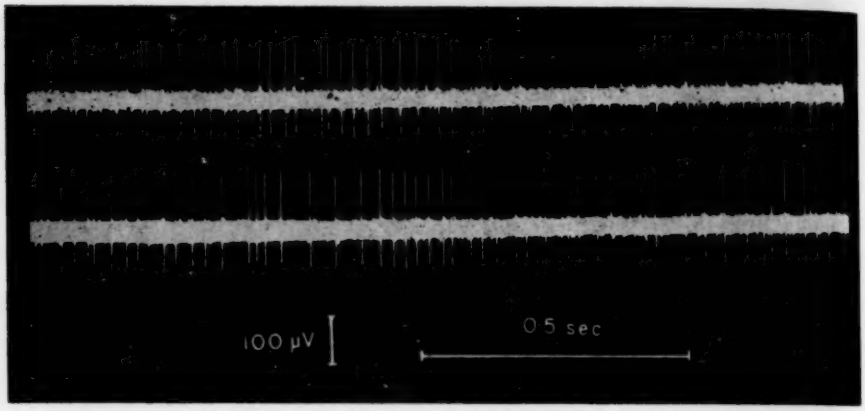

FIG. 6. Maintained discharge in a single off-center ganglion cell, showing fluctuations in the durations of successive intervals. Retouched photographic records.

the minimum interval by inspection of the histogram, and m calculated from the following formula, which can be derived from (1):

$$m = 1/(\bar{t} - d) \tag{2}$$

Here $\bar{t}$ is the mean value of t. The broken curve in Fig. 7, marked "exponential," represents equation (1) as fitted to the data. Chi-square tests showed that all six histograms departed significantly from the exponential curve. (The number of degrees of freedom for the chi-square test was taken as two less than the number of classes into which the data were grouped, since two parameters, d and m, had been fitted to the data.) Assuming for the moment that the exponential curve is the true one, let P be the probability that a random sample would give a worse fit than that actually found; then the smaller P is, the poorer the fit. For unit 1, P was 4.5 per cent; for the other five units, P was 0.1 per cent or less. Since a P of 5 per cent or less is usually taken to indicate a significantly poor fit, all the histograms did not fit the exponential distribution; this shows that, in contrast to the case of the Geiger counter, the probability of the cell's firing during any infinitesimal interval of time dt did depend on the time of occurrence of the preceding impulse, even for intervals much greater than the absolute refractory period (about 1 msec.) of ganglion cells.

Since the histograms were all strongly skewed (asymmetrical about the mean

value) and since the interval t is a positive number, the gamma distribution (Pearson's type III: Kendall, 1945) was tried:

$$f(t) = \frac{k^a t^{a-1} e^{-kt}}{\Gamma(a)}, \quad 0 \leq t < \infty \tag{3}$$

A satisfactory fit was found, although this expression does not contain the minimum interval d. The two parameters a and k were calculated from the

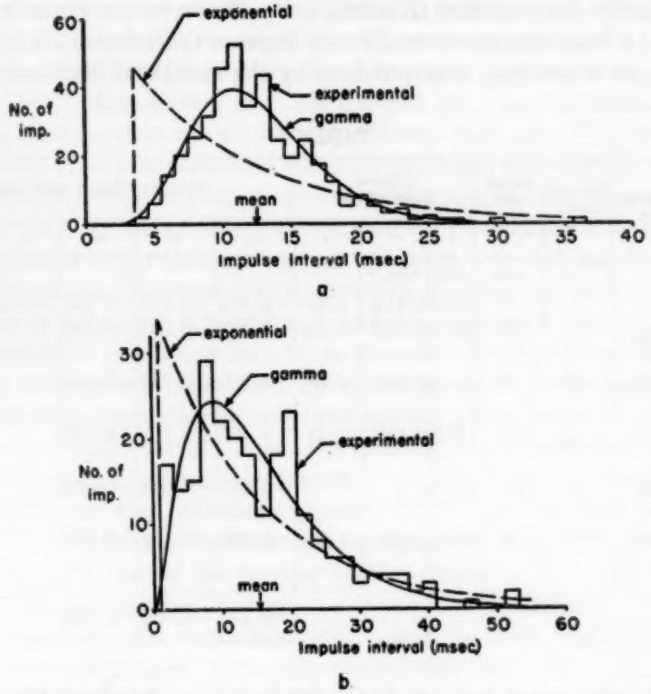

FIG. 7. Two distributions of impulse intervals from different ganglion cells. (*a*) shows unit 3 (see Table I), and (*b*), unit 4. Two theoretical curves, the exponential and the gamma distributions, are shown. Only the gamma gives a satisfactory fit.

experimental mean $\bar{t}$ and the standard deviation σ_t by the following formulas, which can be derived from equation (3) (Weatherburn, 1946):

$$k = \frac{\bar{t}}{\sigma_t^2}, \quad a = \bar{kt} \tag{4}$$

Two of the fitted distributions are shown in Fig. 7, labelled "gamma." Chi-square tests gave values of P between 12 and 70 per cent for five out of the six histograms, indicating good fits. The other histogram (unit 5) had a P of less than 0.1 per cent for both the exponential and the gamma distributions, showing that neither distribution fitted. This histogram was unusually irregular and

not typical, but the same unit had typical serial correlation coefficients (see below).

When the chi-square test was used to test the fit of the gamma distribution, the number of degrees of freedom was reduced by two, as before, since two parameters were estimated from the data. Equations (4), it is true, do not provide a maximum likelihood estimate for the two parameters (as was true for the exponential distribution), although this condition may actually be necessary to justify our procedure (Kendall, 1945). However, any errors in estimating k and a from this source would only increase the value of chi-square and make the fit worse than that provided by the maximum likelihood method.

TABLE I

Unit No.	Type of unit	Retinal illumination	Period of adaptation	Gamma distribution			Serial correlation coefficients				
				$\bar{t}$	k	a	r_1	r_2	r_3	r_4	r_5
		f.-c.	*min.*	*msec.*	*msec.*$^{-1}$						
1	?	?	?	15.7	0.114	1.80	−0.174	+0.046	−0.071	−0.108	+0.050
2	On-center	45	30	25.1	0.163	4.08	−0.117	−0.093	−0.064		
3	On-center	45	7	12.4	0.653	8.08	−0.200	−0.152	−0.037		
4	Off-center	0	48	15.3	0.159	2.43	−0.102	+0.107	+0.019		
5	On-center	0	180	34.2			−0.257	−0.095	+0.036		
6	On-center	0	47	33.5	0.070	2.36	−0.188	−0.122	−0.060		
Mean							−0.173	−0.052	−0.030		

Since, however, except for unit 5, the fits found by the above method were satisfactory, our conclusions remain unchanged.

Table I shows the values of $\bar{t}$, k, and a for the six units (columns 4 to 6). There was no apparent relation between any two of these parameters, and k and $\bar{t}$ did not depend on the state of adaptation (retinal illumination given in column 3), but a was greatest for the two light-adapted units.

The gamma distribution provided a good empirical description of the distribution of intervals in the maintained discharge, but other formulae might have fitted our data just as well. The gamma distribution was chosen partly because it is simple enough mathematically to lend itself to further theoretical studies. Hagiwara (1954), studying afferent discharges from muscle spindles, used a different formula, but his curves look similar to the gamma distribution.

It has been found that the firing probability at any time depends on the

firing times of previous impulses. It is therefore of interest to know whether the firing probability of a given impulse is affected by the times of any impulses earlier than the immediately preceding one. For this reason some of the serial correlation coefficients of the intervals between impulses were computed. The jth serial correlation coefficient r_j is a measure of the correlation between the duration of any interval and that of the jth preceding one (Kendall, 1945; Yule and Kendall, 1950).

The calculated values of some of the serial correlation coefficients are given in Table I. All values of r_1 are negative, and they have a mean of -0.173. This mean value was found to be significantly different from zero by the t-test (Fisher, 1948), which gave a P of less than 0.1 per cent. The mean values of r_2 and r_3, however, were not significantly different from zero ($P = 28$ per cent, 17 per cent). It may be concluded therefore that there is a negative correlation between the durations of pairs of successive intervals, but that for more widely separated intervals we have found no evidence of correlation. This means, for instance, that a longer than average interval tends to be followed by a shorter than average one. Hagiwara (1949), studying the discharge of human motor units during voluntary contraction, found an r_1 of about -0.5; the higher order coefficients were nearly zero. However, in the frog muscle spindle afferent discharge Hagiwara (1954) found no significant serial correlation.

The statistical properties found for the maintained discharge of the ganglion cell may be summarized as follows. The interval durations are not distributed exponentially, and therefore the preceding impulses have an influence on the firing probability that considerably outlasts the absolute refractory state. The gamma distribution, however, fits the data well in most cases. The serial correlation coefficients of the impulse interval durations show that a momentary fluctuation in impulse frequency tends to be compensated for immediately by a fluctuation in the opposite direction, and the firing probability at any time is affected principally by the time elapsed since the immediately preceding impulse, and to some extent by the time between that one and its predecessor, but not significantly by the times of earlier ones. Restated in physiological terms, each impulse is followed by a transient depression of excitability which may outlast the succeeding impulse.

DISCUSSION

Although the existence of "spontaneous" activity in the central nervous system and in sense organs has been accepted for some time, a rigorous proof of spontaneity is difficult to obtain in most cases. Some environmental factors providing "stimuli" are difficult to exclude, even in isolated cells. In sense organs it seems reasonable to apply the term spontaneous to nervous activity which occurs in the absence of a specific or adequate stimulus. An eye which remains in its normal environment and puts out afferent nerve impulses in

the absence of light stimuli thus possesses "spontaneity." Such spontaneity is of special interest in the eye because it related to the threshold-noise problem (Barlow, 1956; FitzHugh, 1957) and may be at the basis of "visual grey" perception.

If a light is shone on the receptive field of an active nerve fiber its discharge may be only transiently changed and in some cases the impulse rate which persisted in the dark may return to its original level. Activity in such a unit during illumination cannot be called spontaneous and therefore the term "maintained," or background, activity is preferred. Furthermore optic nerve fibers which do not alter their discharge rate permanently after changes in illumination, are by themselves not capable of signalling absolute levels of brightness. In other units the frequency changes may be in different directions and would have to be interpreted differently by the brain. In any event since all units seem to be continuously active it is the modulation of the maintained rate in either direction which provides the information of changed background illumination.

We were not able to predict the direction of the change in frequency of the maintained discharge from the discharge characteristics of its receptive field, and we cannot help suspecting that the sensory impulses that convey information about the absolute intensity of the light falling on the eye travel in fibers from units which we have not analyzed. The sustained, reflex constriction of the pupil leaves no doubt that the cat's nervous system receives such information.

Statistical Properties.—A study of the statistical properties of an afferent cell discharge is relevant to two problems: the origin of the discharge in cells and the analysis of the nerve fiber message by the central nervous system.

The first problem has been studied for the afferent discharge from the frog's muscle spindle by Buller, Nicholls, and Ström (1953) and Hagiwara (1954). Some of their interval distributions closely resemble ours. These authors advance the hypothesis that corresponding to each degree of stretch there is a randomly fluctuating level of depolarization of the nerve cell, and that whenever this depolarization rises above threshold, it excites the cell. Following an impulse, the threshold falls from the high value it assumes during the relative refractory period back to its resting value. When it falls sufficiently low, the cell is reexcited. The average interval duration between two successive impulses is determined both by the time course of the recovery curve and by the average level of the stimulating depolarization. The variation in the interval duration is the result of the random fluctuations of the depolarization. In this model no correlation between successive intervals could occur, unless the low frequency components of the noise voltage were so large as to cause a positive correlation. Such a hypothesis thus appears to be too simple to explain the significantly negative values of r_1 for the retinal ganglion cell discharge.

However, if this model were extended to include a temporary decrease of sensitivity following each impulse, a negative serial correlation could occur.

Unfortunately, our statistical analysis enables us only to describe the fluctuating maintained activity in darkness; it cannot tell us where it originates. It might arise either in the photoreceptors, by the spontaneous decomposition of visual pigment, or in an excitation of fine dendritic terminals by random electrical noise of the sort proposed by Katz (1950). Alternately, the self-excitatory process might have a regular rhythm, but be modified by a random process originating elsewhere. For instance, the ganglion cell might have an intrinsic rhythm which is modified by the random arrival of impulses from the bipolars, or the bipolars might all fire with regular rhythms, but at different frequencies and asynchronously, so as to produce a statistically fluctuating state of excitation at the ganglion cell.

The second problem mentioned above, the analysis of the nerve fiber message by the brain, is similar to the engineering problem of detection of a signal in a noisy communication channel. This problem will be treated in greater detail in a separate paper (FitzHugh, 1956), but one aspect of the present data is significant here, namely the negative first serial correlation coefficient (-0.10 to -0.24) between successive impulse intervals. This would make short intervals more likely to be followed by long ones and *vice versa*, and changes in frequency which last for several impulses would be less likely to occur than if there were zero serial correlation. Since such momentary changes in frequency, in the form of short bursts or pauses, characterize the response of a ganglion cell to a short flash, this would appear to be a mechanism making the inherently "noisy" retina detect a flash of light more effectively. However, since the values of the serial correlation coefficients are rather small, this effect may not be very important physiologically.

SUMMARY

Nervous activity has been recorded from the unopened eye of decerebrate cats. Recordings were made with metal electrodes or with small micropipettes from ganglion cells or nerve fibers.

Continuous maintained discharges were seen in all ganglion cells during steady illumination of their receptive fields, as well as in complete darkness. Possible artefacts, such as electrode pressure, abnormal circulation, anesthetic, and several other factors have been excluded as the source of the maintained discharge. Visual stimuli are therefore transmitted by modulating the ever present background activity.

Discharge frequencies were measured following changes of retinal illumination. No consistent patterns of frequency change were found. The maintained discharge frequency may be permanently increased or decreased, or may remain practically unchanged by altering the steady level of illumination. In

addition, there were often transient frequency changes during the first 5 to 10 minutes after changing illumination, before a final steady rate was established.

A statistical analysis of the impulse intervals of the maintained discharge showed: (*a*) the intervals were distributed according to the gamma distribution (Pearson's type III), (*b*) the first serial correlation coefficient of the intervals was between −0.10 and −0.24, with a mean value of −0.17, which is significantly different from zero, (*c*) the higher order serial correlation coefficients were not significantly different from zero. Thus the firing probability at any time depends on the times of occurrence of the two preceding impulses only, and in such a way as to indicate that each impulse is followed by a transient depression of excitability that outlasts the following impulse.

The possible sites at which spontaneous or maintained activity may originate in the retina are discussed.

We are indebted to Dr. K. T. Brown and Dr. T. N. Wiesel for their assistance in many of the experiments and to Dr. P. Meier for his advice on the statistical analysis. We also wish to thank Mr. A. Goebel and Mr. R. B. Bosler for much technical assistance.

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THE VISUAL SYSTEM OF THE ALLIGATOR*

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(Received for publication, December 28, 1956)

The biochemical investigation of visual systems has as yet scarcely touched upon the reptiles. Bliss (1945–46) reported fruitless attempts to extract photosensitive pigments from the lizards, *Sceloporus* and *Xantusia*. This has also been our experience, using much larger quantities of retinal tissue from fresh water turtles (Wald, Brown, and Smith, 1953; unpublished observations). In the retinas of *Pseudemys* we did find vitamin A_2. From retinene$_2$ and cone opsin we also synthesized the photosensitive pigment, cyanopsin. The agreement between its absorption spectrum and the spectral sensitivity of cone vision in the tortoise, *Testudo graeca*, measured electrophysiologically by Granit (1941), implies that cyanopsin is the pigment of cone vision in this animal (Wald, Brown, and Smith, 1953).

In the present paper we report experiments with *Alligator mississippiensis*. The eye tissues and liver of this animal contain vitamin A_1 alone. From the retina we have extracted rhodopsin, typical in absorption spectrum, but synthesized *in vitro* very much more rapidly than the mammalian or frog rhodopsins so far encountered. Electrophysiological measurements of the dark adaptation of the alligator show that this also is very rapid, comparable with the rate of regeneration of rhodopsin in solution.

Vitamin A

The animals were young specimens of *Alligator mississippiensis*, about 20 inches long, obtained from a dealer in Miami, Florida, in August, 1954, and experimented on at once.

Two animals were dark-adapted overnight, decapitated, and the retinas and pigment layers (pigment epithelia and choroid layers) removed under red light. The tissues were ground with anhydrous sodium sulfate until powdery, and extracted twice by stirring with chloroform. Each extract (about 25 ml.) was evaporated to dryness under reduced pressure, and taken up in 1.5 ml. of fresh chloroform. For the antimony chloride test, 1.3 ml. of each of these solutions was mixed with 2.2 ml. of saturated antimony chloride in chloroform, and the absorption spectrum recorded at once in the Cary recording spectrophotometer. The results are shown in Fig. 1.

* This investigation was supported in part by funds from The Rockefeller Foundation and the Office of Naval Research.

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The retinal extract displays two peaks, at about 620 and 660 mμ, due respectively to vitamin A and retinene. (The retinene band, when alone, has λ_{max} 666 mμ; but here is pulled toward shorter wave lengths by its association with the vitamin A band.) This is the result expected if the dark-adapted retinas contained some free

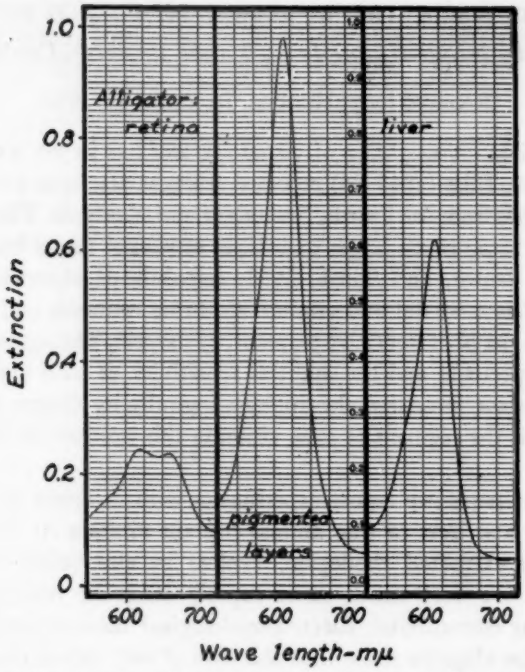

FIG. 1. Absorption spectra of antimony chloride tests with extracts of alligator eye tissues and liver. The retinas and pigment layers (pigment epithelia and choroids) of four eyes were extracted with chloroform, and the whole extracts used in a single test. The retinal extract displays absorption bands at about 620 and 660 mμ, due respectively to vitamin A and to retinene liberated in the destruction of rhodopsin by chloroform. The pigment layer and liver extracts show only the absorption band at 620 mμ due to vitamin A. These spectra were drawn by a recording photoelectric spectrophotometer.

vitamin A in addition to rhodopsin, for chloroform destroys the latter, liberating and extracting retinene.

The extinctions due to vitamin A and retinene in this test can be computed from the extinctions at 618 and 666 mμ, using simultaneous equations. In this way the alligator retina is found to contain 0.171 μg. vitamin A per eye, and 0.525 μg. retinene bound in rhodopsin.

The pigmented layers display the antimony chloride band at 318 mμ due to vitamin A alone. No trace of vitamin A_2 is present. In each eye the pigment layers contain about 1.9 μg. vitamin A.

One liver, of wet weight 4.5 gm., was ground with anhydrous sodium sulfate and

extracted with 60 ml. petroleum ether. An aliquot of this, brought into chloroform for the antimony chloride test, yielded the result shown in Fig. 1. Only vitamin A is present, about 44 μg. per gm. fresh tissue, or about 220 μg. per gm. dry weight.

Rhodopsin

Three animals were dark-adapted overnight and the retinas dissected out in dim red light. They were dropped into 4 per cent alum solution and let harden for 30

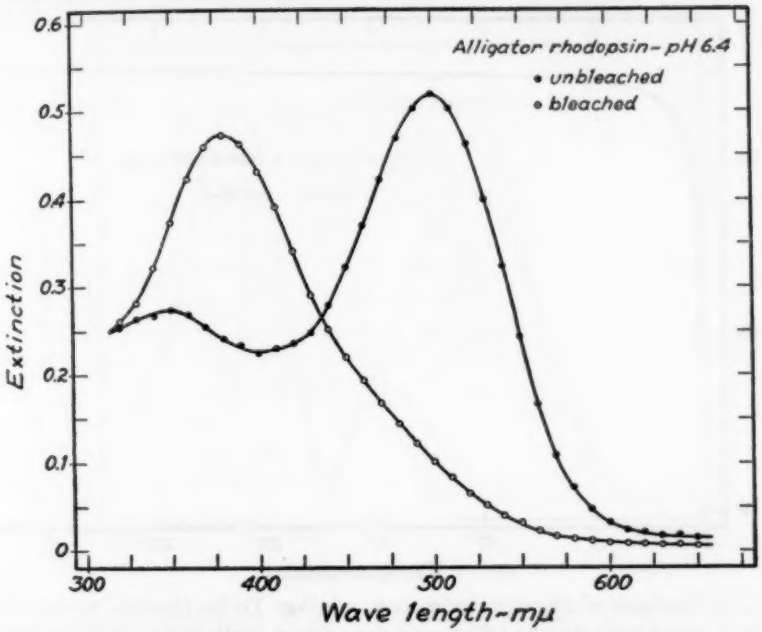

FIG. 2. Absorption spectrum of a solution of alligator rhodopsin in 2 per cent aqueous digitonin, and of the product of its bleaching in orange (non-isomerizing) light (25° C.). The rhodopsin spectrum displays the α-band at 500 mμ, and the β-band at 350 mμ. The product of bleaching is a mixture of all-*trans* retinene and opsin, with λ_{max} 381 mμ.

minutes. They were then washed twice with distilled water and extracted with 2 per cent digitonin in water. The extract was at pH 3.7; it was brought to pH 6.4 with M/15 phosphate buffer, and the absorption spectrum measured. Then this solution was bleached by exposure to deep orange light (the focussed light of a 160 watt microscope lamp passing through a Jena 0G2 filter, which transmits only wave lengths longer than about 540 mμ). The point of using orange light was that this is not absorbed by the retinene which is formed, and hence does not isomerize it. The result of this experiment is shown in Fig. 2.

The alligator retina yielded typical rhodopsin, displaying an α-band with λ_{max} 500 mμ, and a β-band at about 350 mμ. On bleaching, λ_{max} moved to about 381 mμ, and fell somewhat in extinction, results typical of the production of a mixture of all-*trans* retinene$_1$ and opsin at pH 6.4.

Careful tests failed to show the presence of any other photosensitive pigment. One portion of the dark extract was exposed for 20 minutes to the deep red light of the microscope lamp passing through a Wratten 89 filter. An exceedingly slow bleaching occurred, maximal at about 500 mμ, and typical of the bleaching of rhodopsin. This extract therefore contained no appreciable amount of iodopsin or other such red-sensitive pigments as have been found previously associated with cones (*cf.* Wald, Brown, and Smith, 1954; 1954–55).

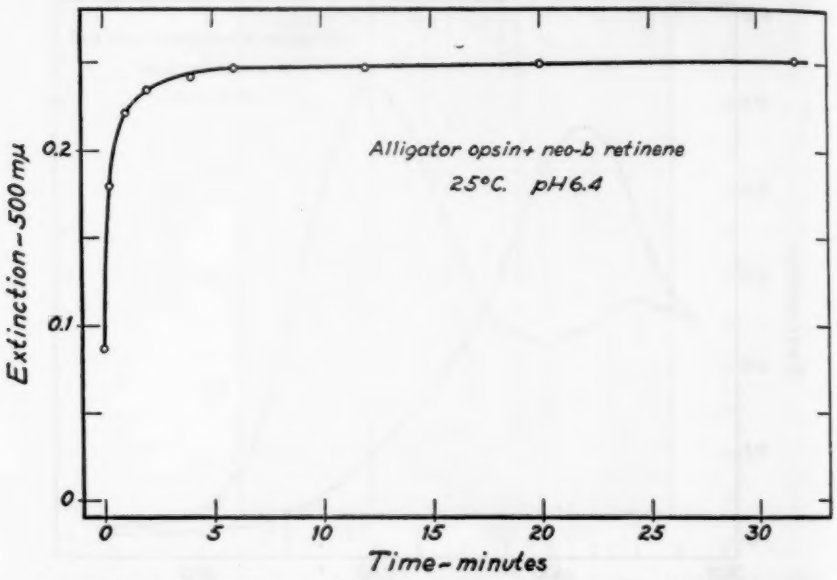

FIG. 3. Synthesis of alligator rhodopsin in solution. To the bleached product shown in Fig. 2, neo-*b* retinene was added, and the mixture incubated in darkness. The extinction at 500 mμ, the λ_{max} of rhodopsin, rose rapidly, reaching close to its maximum value in about 6 minutes.

Synthesis of Rhodopsin

Rhodopsin bleaches irreversibly in orange light to a mixture of opsin and all-*trans* retinene; but is regenerated on adding the hindered *cis* isomer of retinene, neo-*b* (11-*cis*) (Hubbard and Wald, 1952–53; Wald and Brown, 1956). After the bleaching shown in Fig. 2, 0.5 ml. of a solution of neo-*b* retinene was added to 0.4 ml of this extract, and it was incubated in the dark at about 25°C. for 30 minutes. The extinction at 500 mμ rose very rapidly, as shown in Fig. 3, reaching close to the maximum value in about 6 minutes.

At this point 0.1 ml. of a molar solution of hydroxylamine was added, and the absorption spectrum measured in the dark, then after bleaching in orange light. The difference in absorption before and after bleaching (*i.e.*, the difference spectrum) is shown in Fig. 4. It is typical of rhodopsin, with λ_{max} in the visible region at 500 mμ; while in the ultraviolet the extinction rises on bleaching, with λ_{max} 365

mμ, typical of the retinene oxime formed in the presence of hydroxylamine. In two experiments 44.7 and 50.4 per cent of the original rhodopsin regenerated in this way.

Kinetics of Synthesis

The high rate at which alligator rhodopsin is synthesized in solution is a significant datum. The rate of this process far exceeds that of the synthesis of

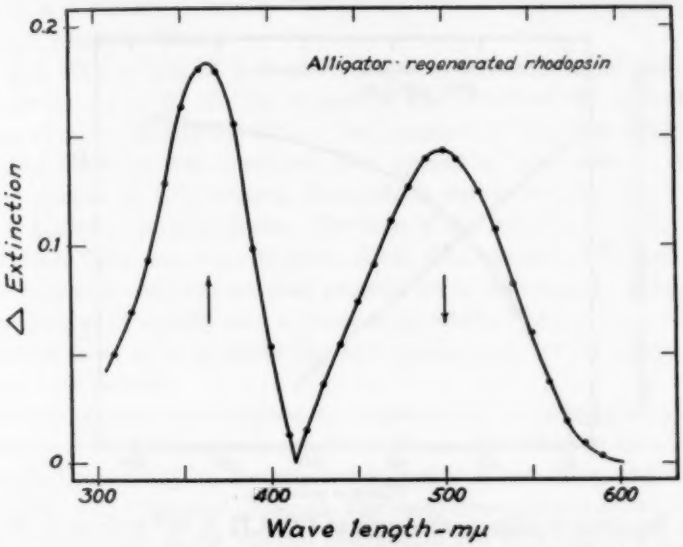

FIG. 4. Difference spectrum of the rhodopsin regenerated in Fig. 3; difference in absorption spectrum before and after bleaching the regenerated rhodopsin in the presence of hydroxylamine, which condenses with the retinene formed by bleaching to yield retinene oxime. The band at the right represents the fall of extinction due to the bleaching of rhodopsin (λ_{max} 500 mμ). That at the left represents the rise of extinction due to the formation of all-*trans* retinene oxime (λ_{max} 365 mμ). 25° C., pH 6.4.

frog, cattle, or chicken rhodopsin. It is indeed too rapid at 25° to permit accurate measurement by the methods we employed; at this temperature the speed of mixing the reagents was an important factor in the first stages of the reaction. For this reason the synthesis was reexamined at 4°C., at which it is greatly retarded (Fig. 5).

It is known that the syntheses of rhodopsin and iodopsin are second-order processes, provided that neither reagent is present initially in great excess (cattle rhodopsin, Hubbard and Wald, 1952–53; Wald and Brown, 1956; chicken rhodopsin and iodopsin, Wald, Brown, and Smith, 1954–55).

The equation of a second-order reaction can be written:

$$t = \frac{2.303}{k(a-b)} \log_{10} \frac{b}{a} + \frac{2.303}{k(a-b)} \log_{10} \frac{a-x}{b-x}$$

in which k is the rate constant, t the time, a and b the initial concentrations of reagents, and x the concentration of product. When $\log (a - x/b - x)$ is plotted against time, this is the equation of a straight line, in which the first term on the right is the intercept constant, and the slope is $2.303/k(a - b)$.

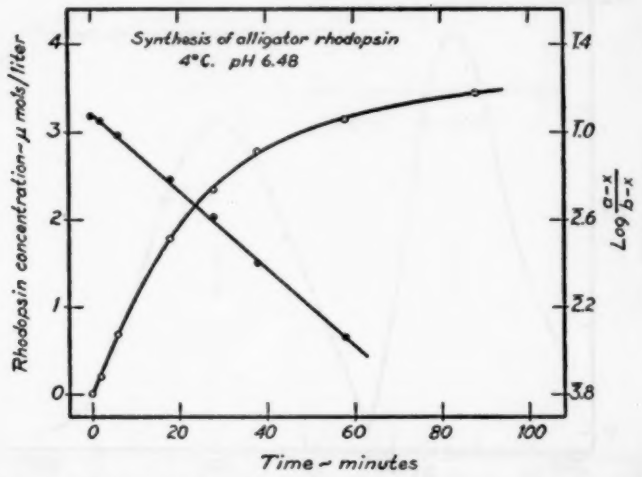

FIG. 5. Synthesis of alligator rhodopsin at 4°C. 1.72×10^{-9} mole opsin and 14.6×10^{-9} mole neo-*b* retinene were mixed in 0.5 ml. of buffered digitonin solution at pH 6.48, and this mixture was incubated in the dark. The open circles show the rise in rhodopsin concentration. The solid circles show values of $\log (a - x)/(b - x)$; that these fall on a straight line is evidence that the synthesis of rhodopsin is a second-order reaction.

In the present experiment, a, the initial opsin concentration, equivalent to the total rhodopsin synthesized, was 3.44 μM/liter; b, the initial concentration of neo-*b* retinene, was 29.2 μM/liter; and x, the concentration of rhodopsin, increased from 0 to 3.44 μM/liter. As shown in Fig. 5, the plot of $\log (a - x/b - x)$ against time (solid circles) is a straight line, from the slope of which k was found to have the value of 0.00159 liters/μM/minute (26.5 liters/mole/second).

The reaction shown in Fig. 5, though bimolecular, has such a large excess of neo-*b* retinene as to proceed almost according to first-order kinetics, its rate depending primarily on the concentration of opsin alone. For this reason the half-time for completion of the reaction may be taken as a rough inverse measure of the rate. The half-time at 4°C. is about 18 minutes, compared with 10 to 20 seconds at 25°C. (Figs. 3 and 6). The unusually high temperature

coefficient implied by this comparison should not be taken seriously until better measurements in the early stages of the reaction can be made at the higher temperature. Such measurements are in progress in our laboratory.

Dark Adaptation

It is often assumed that light and dark adaptation reflect physiologically the bleaching and resynthesis of visual pigments in the rods and cones. Since the synthesis of alligator rhodopsin *in vitro* is very much more rapid than that of the frog, it is of interest to inquire whether the rates of dark adaptation in these animals are similarly related.

The dark adaptations of frogs and alligators were measured under comparable conditions by D. K. The apparatus and details of the procedure are described elsewhere (Kennedy, 1957). The animals were first dark-adapted, and the electroretinogram was measured at a number of intensities of stimulus, using test flashes of 1/50 second, from which curves relating the height of *b*-wave to intensity could be drawn. The form of this function is similar in both animals. From these curves an intensity of test flash was selected which yielded a large response in the dark-adapted animal, yet in the range in which the response still changed rapidly with increase in intensity. The test flash intensities were selected so as to be in about the same position on the intensity-response curves for both animals.

The animals were light-adapted by exposing the experimental eyes to a luminance of 2000 millilamberts for 15 or 30 seconds. Then in darkness they were exposed repeatedly to the selected intensity of test flash, and the ERG was recorded until it became constant. The measurements are shown in Fig. 6, plotted as percentage of the original response of the dark-adapted eye, before it had been light-adapted. In this way the data show the degree of return to the original state, and hence check the condition of the preparation.

It is preferable to make such measurements in terms of the intensity of test flash needed to produce a constant response, rather than following as here the change of response to a constant intensity of stimulus. The latter method had to be used because of shortage of time, and the perishability of the animals during the hot summer weather in which these measurements were made.

Whatever their discrepancies in this regard, these measurements demonstrate clearly that rod vision in the alligator recovers very much more rapidly than in the frog. In the three experiments shown, involving two animals, dark adaptation in the alligator was complete within 5 to 7 minutes, whereas that in the frog took 30 minutes or longer.

The frog dark adaptation is characteristically duplex in form. Apparently the first portion is associated primarily with cone adaptation, the second with rods (*cf.* Riggs, 1937). Two of the three dark adaptation curves measured in the alligator, however, were simple in form, and appear to have been due wholly

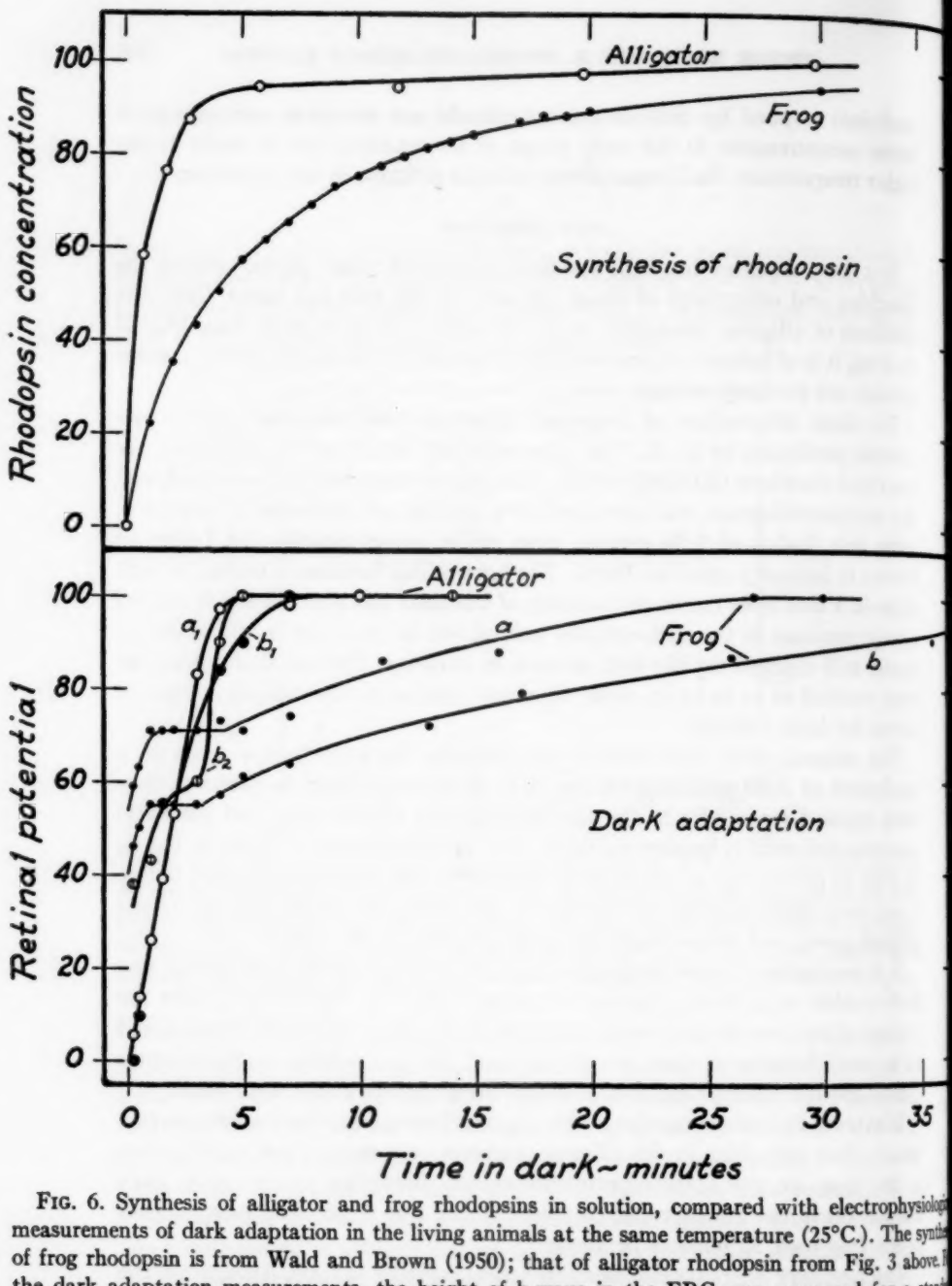

FIG. 6. Synthesis of alligator and frog rhodopsins in solution, compared with electrophysiolog measurements of dark adaptation in the living animals at the same temperature (25°C.). The synth of frog rhodopsin is from Wald and Brown (1950); that of alligator rhodopsin from Fig. 3 above. the dark adaptation measurements, the height of b-wave in the ERG was measured for a stand test flash in the dark-adapted eye. Then the eye was exposed to 2000 millilamberts for 15 seconds or 30 seconds (*b*). Next the eye was stimulated periodically in the dark with the constant test flash, the height of the b-wave recorded as it returned to the dark-adapted condition. This is plotted in lower part of the figure. Data are shown from one frog, and from two alligators (subscripts 1 and 2

to rods. The third of the alligator curves displayed an inflection, owing probably as in the frog, to the participation of cones. Rods and cones have a peculiar distribution in the alligator retina: the entire dorsal portion of the retina, which has a tapetum, is overwhelmingly rod; but ventrad the proportion of cones increases, and at the ventral border cones predominate (Laurens and Detwiler, 1921). The retina as a whole is characteristically rod.[1]

Fig. 6 shows how effectively the cones buffer light adaptation. When cones take little part, as in two of the three alligator curves, light adaptation brings the retinal response to very low values. When cones intervene, as in the third alligator curve and those of the frog, the retina as a whole remains highly responsive to the test flash.

DISCUSSION

As already noted, alligator rhodopsin is synthesized in solution much more rapidly than the rhodopsins of frogs, cattle, or chickens investigated earlier. The latter pigments half-complete their syntheses at 25°C. in 4 to 6 minutes, alligator rhodopsin in 10 to 20 seconds. In this regard alligator rhodopsin resembles the rod pigments—both rhodopsins and porphyropsins—of all the fishes we have so far examined (unpublished observations of P. K. and P. S. Brown). At 4°C., scup rhodopsin is synthesized in solution at about the same speed as the alligator pigment (half-time 5.5 to 6 minutes); and that of the butterfish still more rapidly, with a half-time of only 1.1 minute. Indeed, when brought to the same temperature, butterfish rhodopsin forms in solution with about the same velocity as the cone pigment, iodopsin, from the chicken (*cf.* Wald, Brown, and Smith, 1954–55).

These relationships have an interesting bearing upon the mechanism of dark adaptation. There is good reason to believe that dark adaptation reflects in large part the synthesis of visual pigments in the rods and cones. Indeed the recent extraordinary measurements of Rushton *et al.* have revealed a remarkably close correspondence between the course of bleaching and resynthesis of rhodopsin in the living human eye and the course of human light and dark adaptation (Rushton *et al.*, 1955; Campbell and Rushton, 1955). One has less reason to expect a close correlation between the rate of dark adaptation and the synthesis of rhodopsin in solution from neo-*b* retinene and opsin; for the latter is only the last step in a complex sequence of reactions. Yet this last step may in some cases limit the rate of the entire sequence; and it is of interest to inquire how it compares with the dark adaptation process as a whole.

The present experiments offer a particularly clear opportunity for such a

[1] Walls (1942, pp. 615–616) remarks that in this retina the rods are "rich in rhodopsin and greatly outnumber the cones;" and goes on to say that in the tapetal region the outer segments of the "cones" are "as rod-like as possible (*i.e.*, heavy and cylindrical). Within this single retina we may thus observe a local, partial transmutation of cones into rods."

comparison, for in the frog and alligator a relatively slow and a very rapid synthesis of rhodopsin in solution can be compared with the rates of dark adaptation in the living animals at the same temperature. This comparison is shown in Fig. 6. It reveals an extraordinarily close correspondence between the rates of synthesis of frog and alligator rhodopsin in solution, and the rates of rod dark adaptation in these animals.

In all animals yet observed that possess both rods and cones, the cones dark-adapt much more rapidly than the rods; and in the one case investigated—the chicken—the cone pigment iodopsin is synthesized in solution much more rapidly than rhodopsin (Wald, Brown, and Smith, 1954-55). It would be a mistake, however, to conclude that all cone pigments are synthesized more rapidly than rod pigments, or that all cones dark-adapt more rapidly than rods. Both rod and cone pigments are synthesized in different animals at very different speeds, a property that goes with the fact that they possess different opsins. It is now clear that the rod pigments of some animals (*e.g.*, alligator, fishes) form as rapidly in solution as the cone pigments of others. It appears also to be true that the rods of some animals dark-adapt as rapidly as the cones of others. In any case alligator rods dark-adapt, and alligator rhodopsin is synthesized in solution, at rates comparable with cone dark adaptation and the synthesis of cone pigments in mammals and birds.

SUMMARY

The eye tissues and liver of the alligator contain vitamin A_1 alone. The retina contains rhodopsin, typical in absorption spectrum (λ_{max} 500 mμ); but synthesized in solution from neo-*b* retinene and opsin much more rapidly than are the frog, mammalian, or chicken rhodopsins previously examined. In this regard alligator rhodopsin resembles the rhodopsins and porphyropsins of fishes, all of which so far investigated are synthesized rapidly in solution. The rates of synthesis *in vitro* of frog and alligator rhodopsins are matched closely by the rates of rod dark adaptation in living frogs and alligators, measured electrophysiologically at the same temperature. Alligator rods dark-adapt, and alligator rhodopsin is synthesized in solution, at rates characteristically associated with cones and cone pigments in frogs, mammals, and birds.

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SENSITIVITY TO LIGHT IN THE SEA ANEMONE METRIDIUM SENILE (L.)

II. Studies of Reaction Time Variability and the Effects of Changes in Light Intensity and Temperature*

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(Received for publication, November 23, 1956)

In the first paper of this series it was shown by North and Pantin (1) that the sea anemone *Metridium senile* is not uniformly sensitive to radiation throughout the visible spectrum; maximum sensitivity generally occurred in the blue-green at about 500 mμ, although considerable variability was found from animal to animal. Light and dark adaptation phenomena were also demonstrated.

The entire body surface of *Metridium* is apparently photosensitive, and the animal usually responds to light by local contraction of the parietal musculature; unilateral stimulation, therefore, results in bending, Bohn (2), Parker (3), Batham and Pantin (4). North and Pantin were unable to find either macroscopic or microscopic units suggestive of photoreceptors in the animals, hence the actual site of light perception is still unknown. Both Batham and Pantin and North and Pantin used high light intensities for times up to several minutes. Responses were often movements of a centimeter or more and there was a delay of 20 to 40 seconds between commencement of illumination and the onset of bending. Neither the magnitude of bending, however, nor the time delay were free from variability.

The present investigation deals with much dimmer stimuli and a response of slightly different character. The magnitude of this response is rarely a bending traverse of more than a millimeter; here only bending is involved whereas the high intensity stimuli occasionally cause complete closure, suggesting a more general effect, perhaps even an avoidance reaction. The low intensity response is also involved at high intensities, but may escape notice since it is completed in less than a second with a movement of perhaps a millimeter or less. The action spectra of the two responses are similar, however, and each under the proper conditions displays a reaction time (delay between initiation of stimulus

* Contribution from Scripps Institution of Oceanography, University of California, New Series, No. 918.

and response). Because of its greater uniformity, the present study will utilize only the low intensity response.

Materials and Methods

The illuminator used to supply test stimuli was a model 370 Spencer microscope lamp supplied with a General Electric, vertical ribbon filament, 110 watt projection bulb, type 18AT10/1–6v. The lamp was fitted into a holder containing an additional lens, a 5 cm. plexiglas cell filled with 0.1 M acidic copper sulfate solution, and a slotted holder for 8¼ cm. square filters. The illuminator produced a 1½ × 8 cm. rectangular image of the filament, which was sharply defined at distances from 22 to 35 cm. in front of the forward lens of the system. The input voltage was stabilized by two Sola constant voltage transformers, types CV and CVH, rated at 500 watts each and connected in series. Both transformers were rated to give 1 per cent or better regulation through ±20 per cent fluctuations in line voltage; through the series connection, the regulation was refined to 0.01 per cent. Daily and momentary line voltage fluctuations at the laboratory rarely exceed ±10 per cent.

The regulated voltage was transformed to 6 volts through a triad No. F22-A transformer connected to a variac which controlled the voltage to the illuminator. The latter voltage was kept constant at 6.00 volts by means of a Weston AC voltmeter.

Within the illuminator infrared radiation was removed by a Corning aklo No. 3966 heat filter, 2.5 mm. thick, and by copper sulfate kept in the aforementioned plexiglas cell of 5 cm. pathlength. An Ilford No. 603 spectrum filter, 8¼ × 8¼ cm., made of gelatin, was used to provide blue-green light. This filter had a maximum transmission of 12 per cent at 493 mμ (near the maximum of the anemone's action spectrum) and a transmission of 1 per cent at 473 and 520 mμ; transmissions substantially lower than 460 mμ or beyond 550 mμ were 0.001 per cent or less. Intensities were regulated with Ilford neutral density gelatin filters. All filters were calibrated in a Beckman DU spectrophotometer.

The illuminator was calibrated with the aklo, copper sulfate, and 603 filters in place, by means of an Eppley No. 2470 vacuum thermopile, placed 25 cm. in front of the forward lens of the system, and positioned in the center of the image. The thermopile was calibrated by means of a National Bureau of Standards secondary radiation standard No. C-613, and the output was measured with a Leeds and Northrup type 2284B reflecting galvanometer. Corrections for losses due to absorption, reflection, and scattering by glass surfaces and sea water in the aquarium and aquarium housing totalled 10 per cent and were determined with a General Electric type DW-68 exposure meter.

Throughout the experiments to be described, the animal was illuminated from above by a 2 watt GE 46 radio bulb, 40 cm. distant, shining through an Ilford No. 608 red filter. Voltage across this bulb was regulated as described above. This arrangement cast a general illumination of about 10^{11} quanta/cm.^{2} of light with wave length values greater than 620 mμ upon the surface of the anemone. Light of this dimness and spectral character was assumed to impose no adaptation to the blue-green stimulus. Variation of the intensity of the red light by about threefold did not change the sensitivity of animals to the blue-green stimulus.

The containing aquarium had a capacity of about 10 liters and was kept in a heat-insulated housing in a dark room. The housing was sufficiently large to permit moisture, condensed on the exterior of the aquarium, to be wiped away before each determination. Sea water in the aquarium was circulated continuously by a Jabsco stainless steel pump, through a stainless steel cooling coil and a charcoal column 45 cm. high. Plexiglas tubing was used for interconnecting the components of the circulating system, and water was cycled completely through the aquarium at a continuing rate of about once every 5 minutes. Temperature was controlled by a Fenwall No. 17851 thermoswitch which maintained constancy of $\pm\frac{1}{4}$°C. The entire system was drained and flushed with 40 liters of fresh sea water once or twice weekly. At this time the walls of the aquarium were thoroughly cleaned.

An upright grid of inert black bakelite bars 2 mm. square in cross-section, and arranged to provide 8 mm. square holes, was positioned in the aquarium between the anemone and the eye of the observer. During stimulation the head of the observer was fixed by a rest, the direction of view being perpendicular to the stimulating light beam. Any motion by the animal was readily apparent through the bars of the grid. The anemone was kept on a rotatable, tiltable dais which enabled the observer cautiously to line up the leading edge of the animal with one of the vertical bars of the grid; movements of $\frac{1}{2}$ mm. or less were thus readily detectable. Before each stimulation the anemone was closely observed for at least 30 seconds to ensure that no movement might be in process. If movement was detected, observations were continued until none occurred during a 30 second period. During an experiment the stimulus was extinguished a few seconds after response and at least 5 minutes of darkness intervened between successive stimuli. Measurements of height and diameter were made at intervals during the course of an experiment to ensure that an animal maintained approximately the same shape and volume. Any radical changes in these quantities might alter the total number of photoreceptive units within the illuminated area. Short times (120 seconds or less) were measured with a stop-watch.

Moderate sized specimens of *Metridium* (about 3 cm. diameter and 10 cm. high while expanded) were collected intertidally along the California coast between Pt. Conception and Pt. Reyes. They were allowed to adhere to plexiglas discs and when not under observation were maintained in running sea water cooled to 13°C. Healthy specimens have been thus maintained at La Jolla for 18 months, being fed occasionally on living *Artemia salina*, a small pelagic brine shrimp which *Metridium* readily captures on contact. All data presented were obtained using the white color phase of this anemone, although most of the other color phases described by Fox and Pantin (5) are available. Any variations in sensitivity due to different concentrations of insensitive body pigments were thus minimized.

RESULTS

Variability of the Reaction Time.—The reaction time in *Metridium* (the period elapsing from the instant of illumination until a response is observed) is a feature of great importance, since at any given intensity it is a measure of the number of quanta delivered to the animal. Illumination is apparently necessary for the entire duration of the reaction time; in 70 trials no response was ob-

served if the light was extinguished before the onset of bending.[1] The magnitude of the response (the distance moved during bending) does not appear to correlate with the length of the reaction time.

Unfortunately even under the most uniform environmental conditions obtainable, all animals studied displayed considerable variations in reaction time from one stimulus to the next. Fig. 1 *a* shows a typical record obtained on an anemone from 38 observations during a 7 hour period. Since a light source of constant intensity was used to stimulate, the number of quanta necessary to cause a response varied fivefold in this graph. A sevenfold variation was, in fact, common.

Irregular and large as the fluctuation was, the reaction times of any given anemone always appeared to oscillate around a given average value which did not change over periods of at least a week. It was found, in fact, that when large numbers of observations were studied, statistical similarities among data from different anemones became apparent, and a scheme could be constructed to predict the expected error when only small numbers of observations were available.

If, say, 100 observations were made on an animal, and the reaction times were grouped into classes (for example, out of the 100 observations, 7 might be in the 1.6 to 2.5 second class; 11 in the 2.6 to 3.5 second class; 18 in the 3.6 to 4.5 second class, etc.), a characteristic skewed distribution was always obtained with a degree of kurtosis (peakedness) somewhat greater than ordinarily found in a normal or Gaussian distribution. The anemone data could be adequately fitted by curves calculated from the Poisson distribution

$$F_t = \frac{(Kt)^m e^{-Kt}}{m!}, \tag{1}$$

in which F_t is the frequency of any class of reaction time t, and K and m are constants characteristic of each distribution. The reasons for choosing the Poisson distribution will become apparent below but it should be pointed out that the experimental data could probably be adequately fitted by other distributions as well, such as the Gram-Charlier with a correction factor for kurtosis.

Fig. 1 *b* shows the results of 4 large groups of observations made upon 4

[1] *Metridium* is different in this respect from certain other animals such as *Ciona* and *Mya* (Hecht (6, 7)), *Lampetra* (Steven (8)), or *Cerianthus* (Moore (9)) possessing dermal photosensitivity. The reaction times in the latter group are divisible into a "sensitization period" during which the stimulating light must continue if a response is to be obtained, and a "latent period" during which the animal may remain in darkness and still respond. Both sensitization periods and latent periods might run into several seconds' duration. Any latent period in *Metridium* is probably less than a second, as the animal responds in less than a second to intense flashes of about $\frac{1}{100}$ second duration.

different animals under uniform conditions of environment and stimulus. The data were calculated as cumulative frequency, plotted upon probability paper, and the Poisson distribution of closest fit was drawn. The most obvious difference among the curves is their vertical separation, evidenced by the different values of K needed to fit the curves to the data. This difference suggests that the average reaction time for each animal is different, or the average number of quanta necessary to cause a response varies from animal to animal. North and Pantin (1) have shown that there are factors, such as body pigmenta-

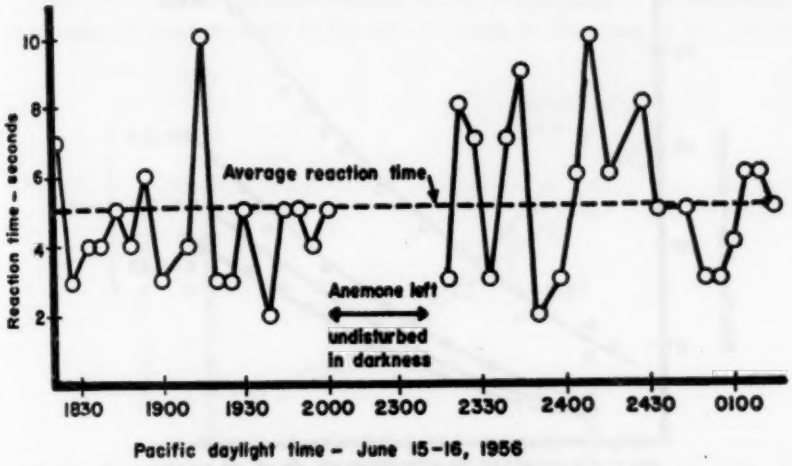

FIG. 1 *a*. Fluctuations in the length of reaction time observed over a 7 hour period; T = 12°C. The points were arbitrarily connected by straight lines. Since any latent period is a negligible fraction of the reaction time, the length of the latter can be used as a measure of the radiant energy necessary to elicit a response. Energy of the stimulus was 8.6×10^8 quanta/cm.^{2} sec. of blue-green light.

tion, that can cause permanent variations in base line sensitivity to blue-green light among individuals of *Metridium*. Undoubtedly there are unknown causes as well, and the existence of such differences is hardly surprising, although well worth future study. The main point to be made here is that the vertical spread of the curves does not necessarily indicate fundamental differences in the patterns of variability found in the animals studied.

Variation of K in Equation 1 involves a slight change of slope in the curves of Fig. 1 *b*, but the top curve is of such different slope that it requires an increase in the value of m to best fit the data. In order to determine whether this might indicate a fundamental difference between this animal and the others, a simple statistical device was constructed to run analyses.

The device consisted of a number of marbles of the same size and weight, placed in an opaque urn. Several colors of marbles were available, and a given color was assigned

to each reaction time class. F_i values for $m = 5$ $K = 1.00$ (see Equation 1) were then calculated, and the number of marbles corresponding to each F_i were placed in the urn. Marbles were withdrawn by touch one at a time, the class noted, the marble replaced, and the urn shaken thoroughly before the next withdrawal.

Ten distributions of 100 withdrawals each were thus obtained, and the two distributions with the most extreme slopes are plotted in Fig. 1 *c*. These ex-

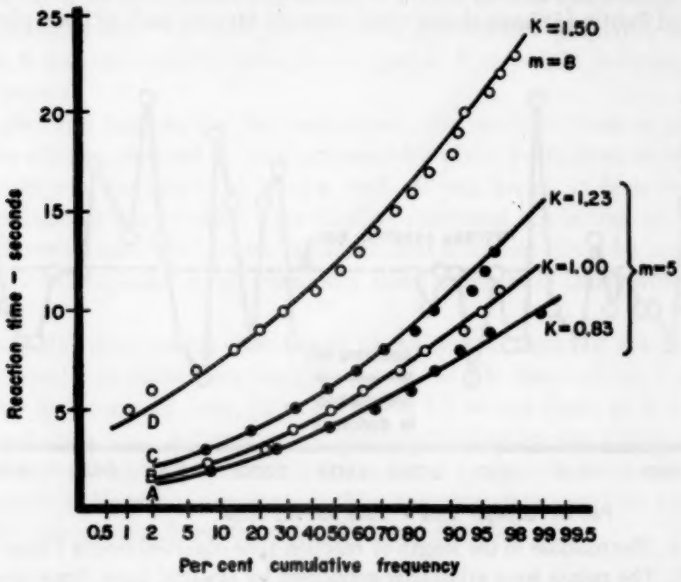

FIG. 1 *b*. Cumulative frequency curves for reaction time distributions gathered from four different anemones at 11.3–12.5°C. The points are experimental data and the lines are calculated values for the Poisson distribution discussed in the text. The record shown in Fig. 1 *a* forms a part of the data used for the points of distribution *A* in this figure. *A* and *D* represent 100 observations, *B* 98 observations, and *C* 81 observations. Energy of the stimulus was as in Fig. 1 *a*.

tremes are closely fitted by curves for which $m = 4$ and $m = 8$, hence it is concluded from the available data that the differences of slope in the curves of Fig. 1 *b* probably have no fundamental significance. A total of eight distributions of 60 or more observations each on eight different anemones have not shown significant deviations from each other when analyzed in the above manner.

It would seem, then, that the patterns of variability in different anemones appear essentially alike when sufficiently large numbers of observations are analyzed. This suggests that the cause of the variability may be a mechanism common to all anemones of this species.

One further question needs to be answered; is there a correlation between the values of consecutive reaction times when series of observations are made, or do the values distribute themselves at random? A rapid and easy way of determining autocorrelation is to count the number of oscillations in a given series of observations. If the values occur at random, the number of maxima and the number of minima found should each be about 1/3 of the total number of observations. Large deviations from the 1/3 value indicate that autocorrelation exists.

To take an example, the record shown in Fig. 1 *a* consists of 38 observations and contains 11 maxima and 11 minima (a point at the start or the end of a

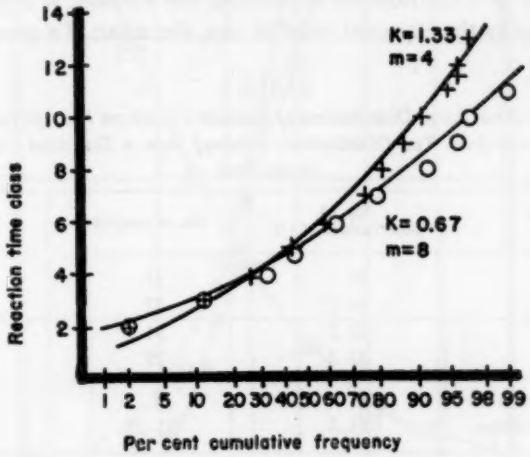

FIG. 1 *c*. Extremes of variation in slope found in 10 distributions obtained from a statistical analyzer described in the text.

series being arbitrarily judged a maximum or a minimum if it deviates from the average by more than 1 second). The series of observations is therefore almost certainly composed of values distributed at random. This conclusion is supported by data obtained from the distributions of Fig. 1 *b*, collected in Table I. In addition, the range of variation is shown in the 10 distributions obtained from the random marble withdrawals which illustrate the variation that can be expected. Our conclusion is that there may be a factor or mechanism in the photosensory processes of *Metridium* which causes as much as a sevenfold random variation in reaction time.

Expected Error for Small Numbers of Observations.—Determination of the large distributions shown in Fig. 1 *b* requires not only considerable time but also a degree of good fortune, since animals may not maintain constant position, shape, or activity for the necessary long periods. Many easier yet significant experiments could be performed, however, based upon smaller numbers of

observations, if the experimenter had a fairly accurate estimate of the errors that might be expected in the procedure he employs. In experiments to be discussed below, for example, it was found necessary to determine the means, the per cent deviation from the mean, and the degree of randomness of small groups of reaction time determinations. A description of the method of determining the expected error in these quantities follows.

It was decided to adopt ten observations as a convenient total for a given series. This number can usually be made in an hour or less, and, as will be seen presently, does not give rise to large expected errors.

Since it has been shown that large numbers of observations of reaction times occur randomly in a distribution resembling the Poisson, it is possible to calculate theoretically the expected error of, say, the mean of a group of 10 obser-

TABLE I

Statistical Data on Four Large Distributions of Reaction Time from Four Different Specimens of Metridium senile and from Ten Distributions Obtained from a Statistical Analyzer Described in the Text

Experiment (Fig. 1 *b*)	No. of observations × 1/3	No. of maxima	No. of minima
C	27	27	28
A	33.3	32	31
B	32.7	31	32
D	33.3	29	29
Range of variation in 10 marble distributions....	33.3	34–28	33–28

vations. It is much less laborious, however, to perform a thousand marble withdrawals in the statistical analyzer, divide them into groups of ten, determine the means of each group, and plot a frequency curve. The validity of this curve can then be checked against results obtained from anemones. Fig. 2 shows the results of such determination of the means, per cent deviations from the mean, and degree of randomness, employing the series of 1000 marble withdrawals used in the previous section. Likewise included in the figures are values obtained from the four anemone distributions shown in Figure 1 *b*. The correspondence is fairly close, and the curves of Fig. 2 will be used in the following section as a basis for estimating the expected error or the expected range of variation when groups of about ten observations each are studied.

Relationship between Reaction Time and Light Intensity.—

Over the range of intensities studied, it has been found that the photosensitive response of *Metridium* obeys, on the average, the Bunsen-Roscoe law of reciprocity

$$I \times t = K, \tag{2}$$

in which I is the intensity of the stimulating light, t is the length of illumination time necessary to elicit response (for the case of *Metridium*, the reaction

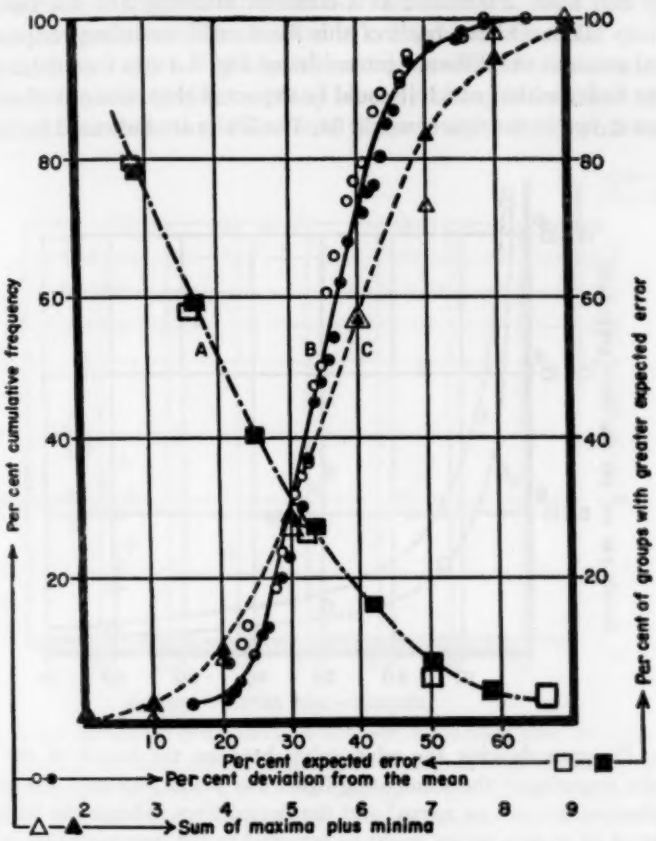

FIG. 2. A. Graph used to determine the expected error in the mean when using groups of ten observations; B. Cumulative frequency curve showing the range of variation to be expected for the per cent deviation from the mean for groups of ten observations. C. Cumulative frequency curve showing the range of variation to be expected for the sum of the maxima and minima found in groups of ten observations. The sum is used as a measure of the randomness within a distribution. Full symbols represent values obtained from marble data, open symbols are values obtained from experimental anemone data.

time), and K is a constant which for *Metridium* appears to be in the neighborhood of 5×10^9 blue-green quanta/cm.^{2}, but varies among individuals by a factor of perhaps three.

Fig. 3 *a* shows the results of 62 observations over a 55 hour period, made on a white animal at different intensities. Each point represents the mean of ten

or eleven consecutive observations. Assuming that the animal obeyed the Bunsen-Roscoe reciprocity relationship, the 62 observations were recalculated as if they had been determined at a constant intensity and the mean of the entire group taken. On the basis of this mean, still assuming reciprocity, the theoretical mean at the different intensities of Fig. 3 *a* was then determined, as well as the limits within which it could be expected that nine out of ten points, such as are shown in the figure, would lie. The limits are indicated by the dotted

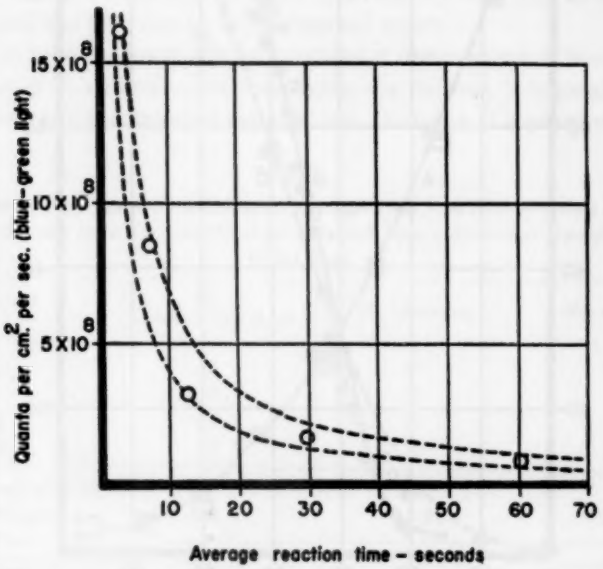

FIG. 3 *a*. Diagram showing the relationship between the length of the reaction time and the intensity of the stimulating light. The points represent the average of 10 to 11 observations on one animal and the dotted lines indicate the limits within which 9 out of 10 of such points might be expected to fall, assuming that *Metridium* obeys the Bunsen-Roscoe law; $T = 12.0°C$.

lines, and it is seen from the points that a satisfactory hyperbolic relationship was obtained between the reaction time and the intensity of the stimulating light. 306 observations on eight different anemones, covering the range shown in Fig. 3 *a*, have produced no evidence to suggest that the reciprocity relationship is incorrect.

We are now in a position to eliminate an uncertainty of Fig. 1 *a*. It will be recalled that in constructing this figure the points were arbitrarily connected by straight lines. Suppose, however, that the fluctuations in reaction time occur much more rapidly and instead of connecting the points with straight lines, two or three or more oscillations between each observation should be

indicated. If such were the case, it would be expected that records made at dim intensities in which reaction times may average a minute or so, would show not only a decrease in the per cent deviation from the mean, but also in randomness, since minor peaks would be levelled as uniform readings.

Fig. 3 *b* shows the effect of change of average reaction time on the per cent deviation and on the randomness. Many of the readings were taken at higher temperatures in the hope that this would increase the number of fluctuations

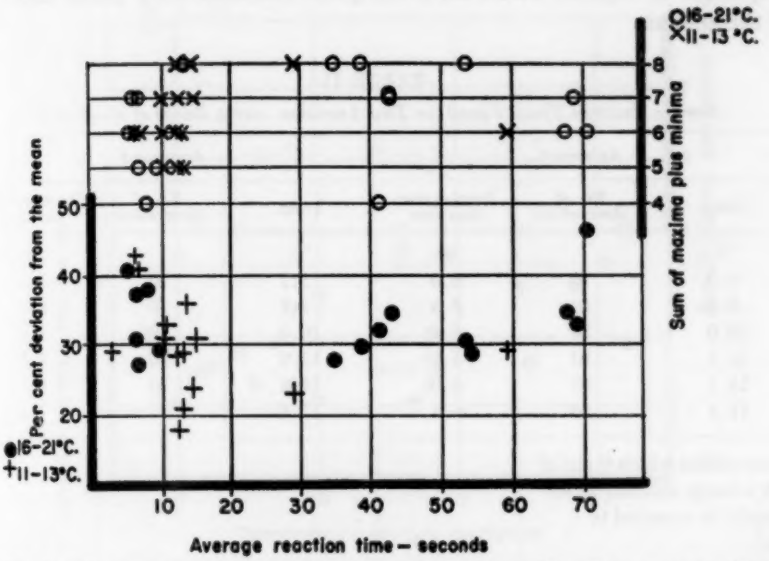

FIG. 3 *b*. Data calculated from 300 observations of reaction time on eight different anemones, showing the distribution of per cent deviation from the mean and of the degree of randomness as a function of the average length of the reaction time. One point (at $\bar{t}$ = 55.1 sec.) was obtained from 7 observations; the rest from 10 to 11 observations.

per unit time and enhance the effect we are seeking, but the next section will show that there is apparently no temperature effect.

There is no marked change in either the randomness or the per cent deviation at longer average reaction times. There is perhaps an unexpectedly large number of values of 8 for the maxima plus minima sum, but in three of the four instances at the longer times they are caused by minor fluctuations such as 52, 54, 51 seconds, which at ten times the intensity would have been recorded as 5, 5, 5, seconds (readings are taken to the nearest second).

The values of per cent deviation at the longer reaction times are slightly low, lying mostly in the second quartile of the frequency curve of Fig. 2. The significance of this will be left for a future investigation, but it is felt that the

slight change does not constitute evidence for a short period fluctuation.[2] We conclude that if irregular fluctuations in the state of the animal are responsible for the observed variability in reaction time, they are probably fairly accurately portrayed by observations made every 5 minutes.

Anemones often require about a minute to respond to light stimuli of 10^9 quanta/cm.^{2} sec. This is roughly the illumination cast by an ordinary street light upon a normal surface about a mile away. About half an hour of dark adaptation is required before an investigator can accurately judge responses by the animals.

TABLE II

Average Reaction Times Found for Two Anemones over a Range of about 12°C

Anemone 1			Anemone 2		
Temp.	No. of observations	Average reaction time	Temp.	No. of observations	Average reaction time
°C.		sec.	°C.		sec.
4.9	10	6.0	5.7	10	7.3
8.4	10	6.3	7.7	10	8.6
10.0	10	6.4	10.0	9	8.4
12.2	10	5.5	12.9	10	8.2
14.1	10	6.0	14.5	10	8.6
17.1	10	6.8	17.5	10	7.3
Limits within which 9 out of 10 average reaction times would be expected to fall.....		5.0–7.4			6.5–9.7

Effects of Temperature upon the Reaction Time.—Table II shows the average reaction times found for two anemones over a range of about 12°C. There seems to be no significant trend or change over this range, which approximately covers the environmental temperatures for the regions where *Metridium* is found along the Pacific coast. A total of 300 observations were made on eight different anemones over the temperature range 4.3—21.0°C., and no significant changes in average reaction time with temperature were found.

As noted above, if a latent period exists in the response of *Metridium* to light it is very short, probably less than a second in duration. A latent period might include thermally affected processes such as nerve conduction time, muscular contraction time, etc., which could conceivably require less than a second to execute. Although muscular contraction is often a slow process

[2] For an oscillation of average wave length 2 minutes or less the per cent deviations should lie between 0 and 18; for an oscillation of average wave length 2 to 4 minutes the per cent deviation should be distributed around 18 as an average.

(Batham and Pantin (10)), *Metridium* can execute a considerable contraction of the longitudinal musculature in a fraction of a second, and conduction in the nerve net of *Metridium* has been shown by Parker (11) to proceed at a rate of about 13 cm./sec. at 21°C. Pantin (12) found similar rates in *Calliactis* for the general nerve net. Photochemical reactions, on the other hand, are well known to be independent of temperature. Since average reaction time does not vary

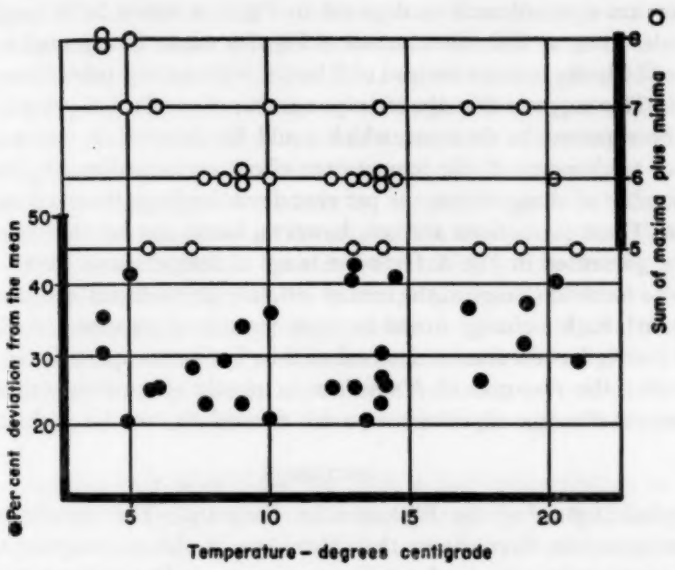

FIG. 4. Data calculated from 290 observations of reaction time on eight different anemones, showing the distribution of per cent deviation from the mean and of degree of randomness at different temperatures. Each point represents 9 to 11 observations. Energy of the stimulus in all cases was 8.6×10^8 quanta/cm.^{2} sec. of blue-green light.

with temperature, it seems safe to assume that most of the reaction time involves photochemical processes.

Lack of a temperature effect upon the length of the reaction time would thus have a plausible explanation. If, however, the variability should arise from changes in physiological state within the animal, one might expect the rate of such changes to display a temperature effect. By way of illustration, suppose that the variability is caused by variance in the tone of the musculature. The body column of a normal *Metridium* is in a state of constant activity, bending about (Batham and Pantin (13)); so a short reaction time might represent an instance when the animal was stimulated just as it was about to execute a forward (*i.e.* "lightward") bend, while a long reaction time might represent an

instance when the stimulus arrived just prior to a backward (or "darkward") bend. The rate of such complex activity should, however, be affected by temperature, and it is indeed found that animals at low temperature are much freer of perceptible random movement and therefore much easier to study. Hall and Pantin (14) found a Q_{10} of 2 for the rate of contraction and relaxation in *Metridium*. The rate of spontaneous bending should, therefore, be considerably retarded at low temperatures. If our hypothesis about muscular tone is correct, the effect on a record such as depicted in Fig. 1 *a* would be to lengthen the time scale. Thus, at 2°C. the abscissa of Fig. 1 *a* might be expected to cover a period of 14 hours or more instead of 7 hours, without any other change in the diagram. This suggests that the average number of oscillations per unit of time would be expected to decrease, which could be detected in our measure of degree of randomness. If the temperature effect were considerable, then, for a small number of observations, the per cent deviation from the mean might also decrease. These predictions are not, however, borne out by the experimental evidence, presented in Fig. 4. Over the range of temperatures shown, at least a three- to fourfold change in the rate of ordinary physiological reactions might be expected. Such a change would decrease the sum of maxima and minima to around 2 or 3, for ten observations taken 5 to 7 minutes apart. It can be concluded that the response of *Metridium* to photic stimulation exhibited no temperature effect in experiments so far devised to test for such an effect.

DISCUSSION

Ecological Aspects of the Photosensitive Response.—The experiments with different intensities have shown that *Metridium* is able to integrate absorbed photic energy over periods of a minute or more, and to utilize the effect to produce a directional response. Several eyeless animals have been shown to possess this ability. Probably the most intensive studies were done on *Ciona* and *Mya* by Hecht (6, 15).

The mechanism of integration of light might conceivably be of advantage to an anemone in a dark environment,[3] causing orientation of the body and tentacles towards steady but weak sources of bioluminescence, thus perhaps increasing the chances of catching microplanktonic luminescent animals for food. Flashes of bioluminescence, presumably from single cells, are often observed in the experimental aquarium, and appear to the human eye many times brighter than illuminations eliciting anemone responses in say, 5 seconds. The ability to integrate radiant energy over the entire body surface might have great potential survival value to other animals as well in the unlighted depths of the oceans where translucency or transparency among organisms is common. Clearly the phenomenon needs to be studied further, not only with regard to

[3] *Metridium* is dredged from depths of 300 to 400 meters in Puget Sound (D. L. Ray, personal communication).

its occurrence, already demonstrated in several phyla, but also with regard to actual uses of it in nature.

Cause of Reaction Time Variability.—Pirenne (16) notes that many investigators have observed variability of response to light in their experimental material when the region of the threshold is approached. In the case of the human eye the source of the variability arises from the uncertainty of quantum capture at dim threshold intensities. For *Limulus*, however, Hartline *et al.* (17) were unable to distinguish between quantal uncertainty and the possibility of variation in nerve thresholds known to occur.

With *Metridium*, for lack of further data, we are at the present time faced with something of the same dilemma, although the current experimental evidence favors the hypothesis of quantal uncertainty. As mentioned above, any variable physiological state such as the tone of a muscle or the threshold of a nerve, depending upon a vast array of physiological and biochemical reactions within the organism, can be expected to display a temperature effect of some sort. In order to explain the observed variability by assuming a fluctuating change in the condition of the animal, we should probably look for a single simple temperature-independent chemical process or, at most, two balancing processes, one with a positive and the other with a negative temperature coefficient. Such processes would need to cause sevenfold changes in photosensitivity of the animal over short periods of time; they have yet to be discovered.

If one assumes that *Metridium* has such a low concentration of photosensitive pigment that only a few quanta may be captured in the brief exposure time at the intensities used, explanation of variability in the reaction times follows. This hypothesis of uncertainty of quantum capture has so far proven entirely adequate as an explanation of the data. Since any latent period is a negligible part of the anemone's reaction time, the latter could be expected to be entirely temperature-independent under this hypothesis. Reaction time distributions would need to conform to the Poisson distribution. And indeed they do, although this is a necessary but not a sufficient requirement. If we assume quantal uncertainty, cumulative curves derived from experimental data have all shown values of m (as defined in Equation 1 above) less than 10, suggesting that the animals responded to less than ten absorbed quanta.

At first thought it seems difficult to conceive that when more than one billion quanta are administered to an anemone, the animal would effectively absorb less than 10 of these. Indeed this is probably not so; measurements upon the body wall of white *Metridium* by North and Pantin (1) have shown transmissions of roughly 50 per cent at 500 mμ. Of the remaining 50 per cent a large fraction is undoubtedly reflected, scattered, or otherwise dispersed, but nonetheless considerably more than 10 quanta are probably absorbed by the

tissues. It must be remembered, however, that there is apparently a specific pathway by which absorption of quanta must occur and still elicit a response by the animal; action spectra for *Metridium*, with maxima lying between 500 and 600 mμ, have been found by North and Pantin (1), suggesting that quanta must be absorbed by a certain pigment or perhaps a few pigments. The concentration of such a photosensitive pigment, therefore, is the critical factor that must be considered.

Unfortunately hardly a beginning has been made towards isolating possible photosensitive pigments from *Metridium*. Indeed if we assume that the hypothesis of quantal uncertainty is correct, even a rough calculation quickly indicates the futility of attempting to isolate such a pigment in any reasonable quantity, much less determine the concentration accurately[4]. We note

[4] Let us assume, as a rough conservative estimate from the data presented above, that on the average 1 quantum in 10^9 is absorbed by the photosensitive pigment in the illuminated area of the animal. Assume, further, a quantum efficiency of unity and let the concentration of pigment be c, its molar extinction coefficient at the wave length of maximum absorption be E, and assume that the thickness of the body wall of the anemone is 10^{-1} cm. Then

$$c = \frac{ln(I_o/I)}{E \times l} = \frac{ln\left(\frac{10^9}{10^9 - 1}\right)}{E \times 10^{-1}} = \frac{10^{-8}}{E}, \text{ expressed as moles/liter}$$

In order to obtain a fairly good absorption spectrum in a Beckman DU spectrophotometer, with a 1 cm. path-length cell, the concentration of pigment should be such as to render the maximum per cent absorption not much less than 10 per cent. Such a concentration of pigment would be

$$c = \frac{ln(100/90)}{E \times 1} = \frac{10^{-1}}{E},$$

again expressed as moles/liter. A concentration factor of 10^7 is therefore indicated.

An average sized *Metridium* contains perhaps 10 cc. of tissue, therefore, one million such animals would be expected, according to our calculation, to yield 1 cc. of dilute extract for spectrophotometric analysis, assuming no losses during the extraction procedure. To conduct such an experiment in California would require several man-years of effort just to collect the necessary anemones; the chemical extraction might well be complicated by similar but non-photosensitive pigments which would almost certainly be present in the 100 tons or so of material. To attempt the collection and analysis hardly seems practical.

The hypothesis of quantal uncertainty can be shown to lead to sensible results if we assume a value of E in the equation above, of 10^5, a value common among carotenoids at the wave length of maximum absorption. It can then be calculated that our average sized *Metridium*, fully expanded, would have 10^6–10^7 photosensitive pigment molecules per cm.^{2} of body surface. The animal would need perhaps 10^{14} of such molecules per cm.^{2} before it would appear faintly colored.

in passing that *Metridium* may represent a biological extreme in photosensitive machinery. On the one hand we have receptors such as the human eye, where evolution has conveniently concentrated the photosensitivity pigment. Threshold studies here must deal with the uncertainty of the exact number of quanta contained in any stimulating flash. In similar studies with *Metridium*, on the other hand, the number of quanta per stimulus is readily determinable with sufficient accuracy, but the uncertainty element may still be present, since the pigment concentration may be so dilute as to render any adequate determination of it all but impossible.

In retrospect, the critical data that support the hypothesis of quantal uncertainty are the variabilities in reaction time and especially the demonstration of a complete lack of a temperature effect. At the same time the length of the reaction time is intimately related to illumination since it can easily be varied by changing the intensity of the stimulus. While these considerations constitute strong arguments for the hypothesis, they are perhaps not the conclusive proof that is needed for complete acceptance. In dealing with the properties of such a dynamic fluid as protoplasm, of which we possess only a fragmentary knowledge, it would seem wise to view with reservation conclusions dependent upon indirect evidence.

SUMMARY

1. The reaction time of the photosensitive response of *Metridium* was found to be composed almost entirely of a sensitization period. If a latent period exists, it is too short to be detected by the methods used and is probably less than a second in duration. The length of the reaction time, therefore, was used as a measure of the radiant energy necessary at any intensity to elicit a response.

2. The length of the reaction time was found to vary randomly by a factor of seven under constant environmental and stimulating conditions. Determination of large numbers of reaction times on several anemones, grouped according to length, gave closely similar distributions resembling Poisson distributions. It was suggested that the variability may be caused by the same factor or mechanism in each individual. An experimental scheme was presented for determining the expected error and variation in statistical quantities when groups of ten observations are used.

3. The means of groups of reaction times determined at different intensities formed a hyperbolic relationship when plotted against intensity, suggesting that the animal obeys the Bunsen-Roscoe law of reciprocity. No marked changes were noted of per cent deviation from the mean or of randomness at different intensities. Stimulation of *Metridium* requires roughly 5×10^9 incident quanta/cm.^{2} of blue-green light.

4. No temperature effect could be found on either the means, the per cent

deviation from the mean, or the degree of randomness of series of about ten observations, studied over a 17°C. range. It was concluded that the variability in reaction time was not due to changes in any complex physiological state such as muscular tone.

5. A possible use of the photosensitive response is suggested and the potentialities of "integrative" photosensitivity are discussed.

6. Possible mechanisms to explain the variability in reaction time are discussed. In the light of evidence presented, the most likely hypothesis appears to be the uncertainty of quantum capture caused by low concentrations of photosensitive pigment. Assuming the validity of this hypothesis, evidence suggests that the anemones responded to less than 10 quanta of absorbed light. Caution, however, is recommended in accepting the hypothesis because of the indirect nature of the supporting evidence.

Grateful acknowledgment for helpful criticism and advice is made to Drs. Brian Boden, Frederick Crescitelli, Carl Eckart, Denis L. Fox, Francis T. Haxo, Elizabeth Kampa, Leonard Liebermann, C. F. A. Pantin, and W. A. H. Rushton, and for aid in collecting specimens to Messrs. Raymond J. Ghelardi and Conrad Limbaugh.

It is a pleasure to acknowledge the support given by the George Allen Bisbee Donation and The Rockefeller Foundation Donation for Research in Marine Biology.

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STEPS IN THE PRODUCTION OF MOTONEURON SPIKES

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(Received for publication, November 29, 1956)

The first report on action potentials recorded with intracellular electrodes from motoneurons (Brock, Coombs, and Eccles, 1951) described orthodromic and antidromic spikes as identical except for presence of a synaptic potential at the foot of the spike evoked by orthodromic stimulation. It was later recognized (Brock *et al.*, 1952 *a* and *b*) that a difference exists in the rising phase of spikes evoked by orthodromic or antidromic stimulation, and it was stated that antidromic spikes show an initial fast rise of about 35 mv., an inflection, and a further fast rise to peak, while the orthodromic spikes present an initial slow rise of about 10 mv. (the synaptic potential) an inflection there, and a further fast and smooth rise to peak. It was then suggested that ". . . the spike response of the neurone began at the inflection on the rising phase. . . . In the former [antidromic] the inflection indicates origin of the neuronal spike from the spike of the non-medullated axon, in the latter [orthodromic] its origin from the synaptic potential" (Brock *et al.*, 1952 *a*, p. 440). This view was further developed in a later article (Brock *et al.*, 1953) in which an attempt was also made to justify the difference of depolarization level required for initiation of soma firing following orthodromic or antidromic stimulation.

Araki, Otani, and Furukawa (1953) working with toads noted that an inflection is often present also in the rising phase of orthodromically evoked spikes, and Araki and Otani (1955) recorded a similar inflection also in spikes evoked by direct stimulation of toad's motoneurons. They interpreted these findings by assuming that ". . . the non-myelinated segment is more excitable than the soma, so that the local current due to synaptic potential may excite it primarily, as suggested by Gesell" (Araki and Otani, 1955, p. 475). Frank and Fuortes (1955 *b*) confirmed the presence of an inflection in the rising phase of orthodromic spikes and showed in addition that orthodromically evoked spikes may be made to fall from the point of inflection just as occurs for antidromic

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spikes. These results were interpreted at the time by assuming that orthodromic impulses normally originate in dendrites and are conducted from there to the soma. The inflection was therefore tentatively ascribed to delay of conduction between dendrites and soma similar to the delay of conduction between axon and soma previously postulated.

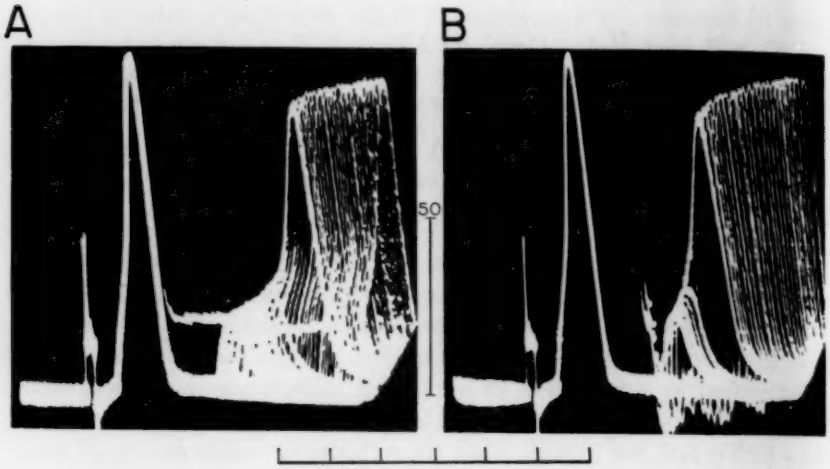

FIG. 1. Spikes evoked in the aftermath of an antidromic spike. Superimposed sweeps. Calibration, 50 mv. Time, 1 msec.

A. Antidromic stimulus followed at different intervals by a second antidromic stimulus. The second stimulus evokes only a small spike at stimulus intervals less than 2.6 msec. and no spike at all when stimulus interval is less than 1.8 msec.

B. Antidromic stimulus followed at different intervals by a brief direct stimulus. Strength of direct stimuli constant throughout. The direct stimulus evokes a small spike only at (corrected) stimulus intervals less than 2.3 msec. and no spike at all at less than 1.8 msec.

The present study is an attempt to analyze the experimental conditions controlling the described features of motoneuron spikes with the aim of understanding their mode of production. The analysis has been performed systematically for spikes originated by antidromic or direct stimulation but the study of synaptically evoked spikes has been limited to few incidental observations.

Methods

This research was performed on cats anesthetized with pentobarbital sodium (nembutal). KCl-filled micropipettes were inserted in spinal cord motoneurons and used both for leading off potential changes and for applying electrical stimuli directly to the penetrated cells. Reference is made to two previous papers (Frank and Fuortes, 1955 *a* and 1956) for details of the methods used for preparing the animal and for stimulating and recording.

RESULTS

Spike Evoked in the Aftermath of a Preceding Spike. Motoneuron spikes evoked by antidromic excitation or direct stimulation through the microelec-

FIG. 2. Tracing of spikes evoked by direct stimuli in aftermath of an antidromic spike. As stimulus interval was decreased, strength of direct stimulus was increased to the intensity which would elicit either a small or a large spike. Level of transition between small and large spike is raised as stimulus interval is decreased. Time, 1 msec.

trode change their features if they are evoked within a short time after a preceding spike. The changes occurring in antidromic spikes have already been described (Brock *et al.*, 1953; Frank and Fuortes, 1955 *a*) and are again illustrated in Fig. 1 A. Fig. 1 B shows that similar changes occur when a spike is evoked by brief direct electrical stimuli of constant strength applied at different

times after a spike of antidromic origin. In either case the height of the second spike decreases gradually and the inflection becomes more prominent as the second stimulus approaches the first. At an interval previously referred to as "critical stimulus interval" (Frank and Fuortes, 1955 *a*) the second spike fails to develop fully and falls instead from the point of inflection, thus leaving a small spike only. This small spike further decreases in size and duration as

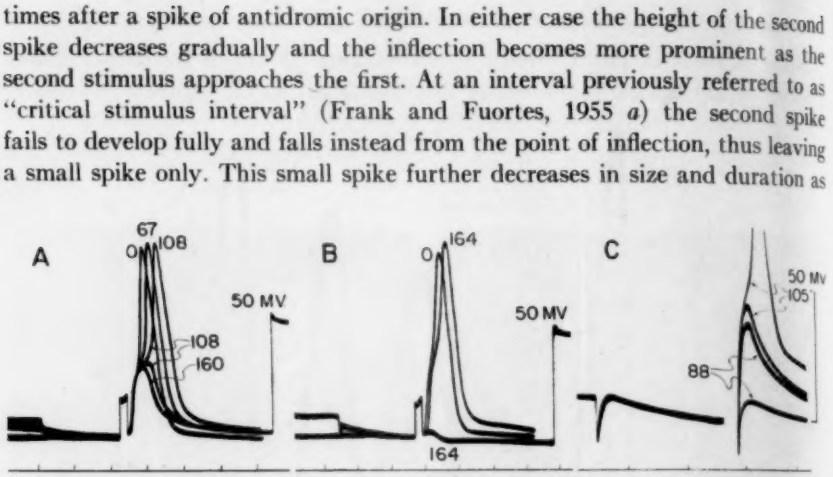

FIG. 3. Tracings of spikes evoked during hyperpolarization. Calibration, 50 mv Time, 1 msec.

A. Antidromic spikes evoked during flow of hyperpolarizing currents of different intensities (indicated in arbitrary units). At current intensity 108 antidromic stimuli may elicit either a full spike with a prominent inflection or a small spike only. Both peak height and area of small spike decrease with current of higher intensity (160). Note that since polarizing currents are applied through a bridge circuit (*cf.* Frank and Fuortes, 1956) baseline positions have no relation to actual membrane potential, but are adjusted to superimpose at the time of antidromic stimulation. Square wave at end of sweep is the 50 mv. calibration.

B. Experiment as in Fig. 3 A, but on another unit. In this experiment, when intensity of hyperpolarizing current is 164 the antidromic stimulus may evoke a full spike or no spike at all.

C. Direct spikes evoked during hyperpolarization. Strength of hyperpolarizing current pulse constant throughout. The numbers indicate strength of direct stimuli in arbitrary units. Stimulus strength 105 may evoke either a (larger) small spike or a full spike.

stimulus interval is shortened, until no spike at all can be elicited by the stimulus used. The tracings of Fig. 2 illustrate the results of an experiment in which not only the timing relative to a preceding spike but also the strength of the direct testing stimulus to a motoneuron was changed. Critical stimulus interval was about 7 msec. for the weakest stimulus used. For stronger stimuli, the critical interval was shortened while transition from small to full spikes was shifted to higher potential levels.

Spikes Evoked during Hyperpolarization of the Motoneuron Membrane. Some of the changes occurring in antidromic spikes when the motoneuron membrane is hyperpolarized have been mentioned in a previous article (Frank and Fuortes, 1956). These changes are illustrated in Fig. 3 A which shows that, with in-

creasing polarization, the notch in the rising phase becomes more prominent and "partial block" may occur. The same results are obtained if a spike is evoked by brief direct stimuli while the motoneuron is hyperpolarized. When direct stimulation is used, stimulus strength can be changed at will. It is seen then that on the background of a constant hyperpolarization a weak stimulus may give rise to a small spike only, while a full size spike may be obtained

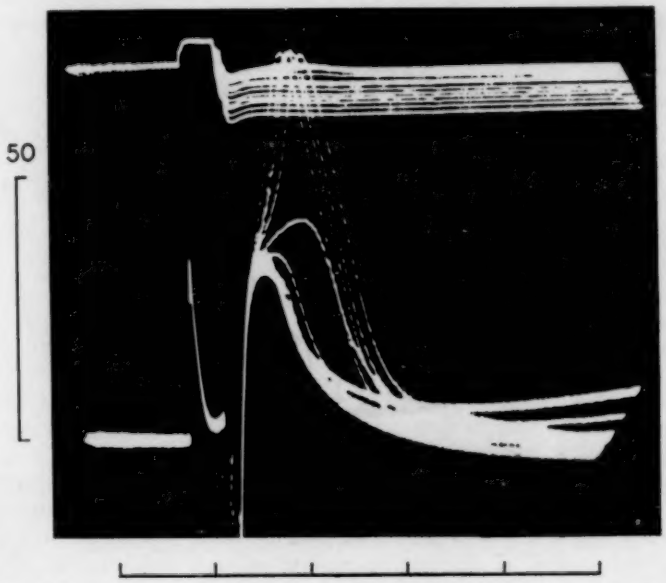

FIG. 4. Spikes evoked by diphasic direct stimuli. Stimulating current, indicated by upper beam, consists of a brief depolarizing pulse of constant intensity, followed by a longer hyperpolarizing pulse of different intensity in the individual sweeps. As hyperpolarizing current is increased, inflection in rising phase of spike is made more prominent until a small spike only is evoked. Calibration, 50 mv. Time, 1 msec.

with stronger stimulation (Fig. 3 C). Whereas partial block can always be obtained in the aftermath of a spike (Fig. 1), in some units it is not obtained with continuous hyperpolarization. The effect of hyperpolarization on units of this type is illustrated in Fig. 3 B in which it is seen that, at a critical level of hyperpolarization, antidromic stimuli evoke either a full spike or no spike at all.[1] This result indicates that the region of the motoneuron responsible for

[1] A similar "total block" has been evoked by Brock *et al.* (1953) with high frequency repetitive stimulation. They recorded then a small (1 mv.) deflection (their "M" spike) which they ascribed to activity of the myelinated axon. In the present study, small potential changes were often contributed by activity of elements surrounding the penetrated unit, and no attempt was made to distinguish these from similarly small changes due to activity of the impaled cell.

generation of the small spike is close enough to the microelectrode tip to be readily affected by currents applied through it. However, partial block of a spike can always be obtained with hyperpolarization if the hyperpolarizing current is applied after the spike has been initiated, as illustrated in Fig. 4.

Spikes Evoked in the Aftermath of a Small Spike.—The results mentioned so far appeared to be compatible with the assumption that the two components of motoneuron spikes result from activity of different portions of the motoneuron and other experiments were designed with the aim of testing this assumption and perhaps of establishing how different areas of a motoneuron participate in the processes of excitation and conduction.

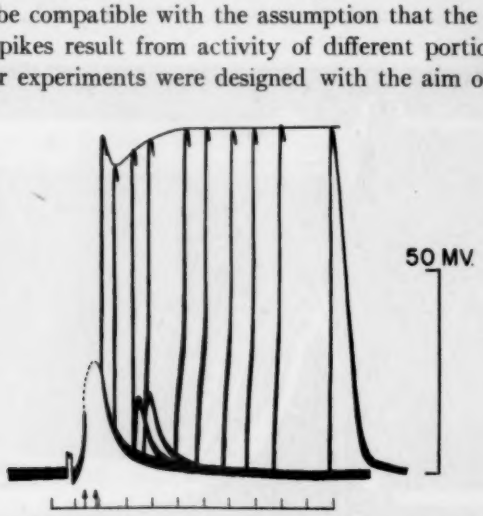

FIG. 5. Spikes evoked by direct stimuli in the aftermath of a small spike. The first small spike is evoked by the combination of an antidromic stimulus (indicated by the artifact) and a brief hyperpolarizing pulse (indicated by the arrows). Strength of direct stimuli applied at different intervals after small spike was adjusted to be just threshold for firing (*cf.* Fig. 9). At stimulus intervals between 2 and 3 msec. weak stimuli elicited small spikes only and stronger stimuli elicited full spikes. Calibration, 50 mv. Time, 1 msec.

It will be convenient to call the region of the motoneuron responsible for production of the small spike "A," and the region responsible for production of the later phase of the spike "B."

If the small spike is followed by a period of absolute refractoriness of the area responsible for its generation ("A"), it might be possible to elicit in isolation the spike due to the firing of other portions of the motoneuron ("B"). If the small spike is the result of activity of one area of the motoneuron, then, with simple assumptions, it might be expected that activity of the remaining area in isolation would also give rise to a spike of reduced height. Therefore, the experiments described in this section and illustrated in Figs. 5 to 7 were designed to analyze the features of spikes elicited in the aftermath of a small

spike. In the experiment of Fig. 5 the conditioning small spike was elicited by blocking an antidromic spike with a brief hyperpolarizing pulse while in the experiment of Fig. 6 the small spike was elicited by the second of two antidromic stimuli within the critical stimulus interval. In both cases a direct testing stimulus of constant strength was delivered at different times relative to the small spike. For the longer stimulus intervals, the responses to the test shocks are similar to those occurring following a full spike shown in Fig. 1 B; as stimulus interval is decreased, the spike evoked by the direct test stimulus

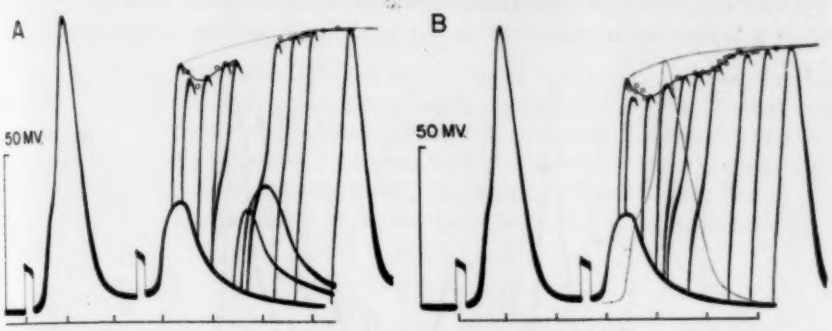

FIG. 6. Spikes evoked by direct stimuli in the aftermath of a small spike. The small spike is evoked by the second of two antidromic stimuli, indicated by the artifacts. The following spikes are evoked in A by weak and in B by stronger direct stimuli applied at various intervals after the second antidromic stimulus. Strength of direct "test" stimuli is kept constant throughout each experiment. Note increased prominence of rising phase inflection with block possible at a critical stimulus interval (A) and absence of visible inflection at shorter intervals. Broken tracing in B shows one response to test shock when second antidromic conditioning shock is omitted. The upper broken line plots the peaks of such responses (see Fig. 1). Calibration, 50 mv. Time, 1 msec.

presents a more and more pronounced inflection and may critically change into a small spike (Figs. 5 and 6 A). (As seen in Fig. 6 B, using slightly stronger test shocks this partial block does not occur.) At very short stimulus intervals the test stimulus again evokes a larger spike and this does not present any visible inflection. The solid lines in Figs. 5 and 6 indicate peak height of the potentials generated by the combination of stimuli used, and the dotted lines the height of the potentials evoked by the direct test stimulus in the absence of the small conditioning spike.

The responses which do not present a rising phase inflection and which occur when the "A" area is presumably refractory can be ascribed to activity of the non-refractory "B" area of the motoneuron. "B" spikes evoked in this way cannot be recorded in isolation because they can be elicited only during the falling phase of the "conditioning" "A" spike.

Fig. 7 illustrates the results of an experiment in which an antidromic shock was used as a test stimulus. In this case, partial block occurred at a critical interval and, for shorter intervals, the small spike further decreased in size, until no spike at all could be elicited.

These results show that the non-refractory area of the motoneuron can be reached by stimuli applied through the microelectrode, but not by antidromic stimuli.

Excitability Following a Full Spike.—All results quoted so far agree in showing that in a variety of conditions it is easier to elicit a small than a large spike from a motoneuron. According to the working hypothesis mentioned above,

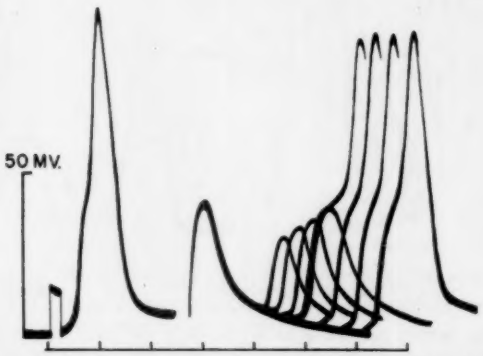

FIG. 7. Spikes evoked by antidromic stimuli in aftermath of a small spike. The conditioning small spike is evoked by a direct stimulus following an antidromic stimulus. The subsequent antidromic test stimuli evoke either a notched spike or a small spike only and no spike at all is elicited by antidromic stimuli applied at less than 1.5 msec. after initiation of the first small spike. Calibration, 50 mv. Time, 1 msec.

this means that firing of the "A" area of a motoneuron may be elicited by stimuli which, in those conditions, are incapable of eliciting firing of the whole motoneuron. Analysis of this phenomenon was approached by means of excitability studies to determine the relative strengths of the stimuli required to evoke partial or total firing of a motoneuron under various circumstances. In normal conditions a short direct stimulus to a motoneuron has an all-or-none effect evoking either a full spike or no response at all, while if a stimulus is applied shortly after firing of an impulse a small spike only may be evoked. Fig. 8 shows the strength of the stimuli required to evoke a small or a full spike at various times after firing of an antidromic spike. It is seen that, when weaker stimuli evoke a small spike only, a full spike can still be evoked if stimulus strength is increased. It will be noted that the late excitability changes illustrated in Fig. 8 appear to correlate with the afterpotentials of the spike. In the majority of the units studied both afterpotentials and excitability changes

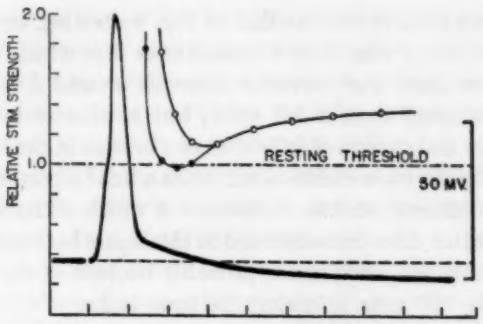

FIG. 8. Excitability following a full spike of antidromic origin. Ordinate, strength of threshold test stimulus expressed as a fraction of resting threshold. Abscissa, time in milliseconds. Filled circles indicate height of stimuli producing a small spike only and open circles indicate height of stimuli on the same time scale evoking a full spike. The conditioning antidromic spike is traced to show time relation of excitability changes to the spike itself and to its afterpotentials. 50 mv. calibration applies to this tracing.

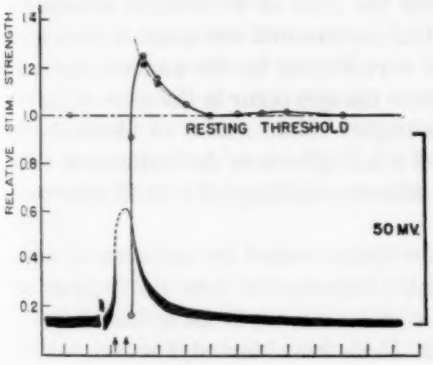

FIG. 9

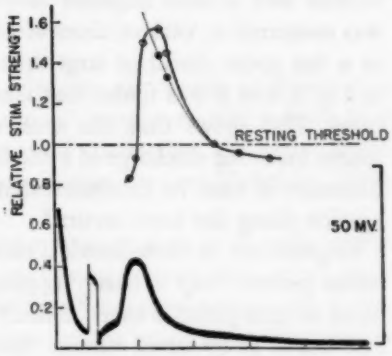

FIG. 10

FIG. 9. Excitability following a small spike. Small spike was evoked by the combination of an antidromic stimulus and a strong hyperpolarizing pulse (indicated by arrows). The experimental data are plotted as in Fig. 8. 50 mv. calibration refers to spike tracing.

FIG. 10. Excitability following a small spike. Experiment similar to that illustrated in Fig. 9, but small spike was evoked by the second of two antidromic stimuli, as in the experiments illustrated in Fig. 6. 50 mv. calibration refers to tracing.

were the same following an antidromic or a direct spike. In other units both hyperpolarization and excitability changes were larger after antidromic than after direct spikes presumably as a consequence of activation of motoneuron axon collaterals (Renshaw, 1946; Eccles, Fatt, and Koketsu, 1954).

Excitability Following a Small Spike.—If an antidromic spike is partially blocked by a brief hyperpolarizing pulse so that a small spike only occurs, then

excitability follows a curve such as that of Fig. 9 showing, for decreasing stimulus intervals, at first a rise, then a conspicuous dip, which is a mirror image of the small spike itself. For stimulus intervals around 2 msec. it is slightly easier to evoke a small than a full spike, but at shorter intervals spike size suddenly increases and no sign of inflection can be seen in the rising phase (Figs. 5 to 7). Essentially the same events occur when a brief direct stimulus is applied after a pair of antidromic stimuli, the second of which elicits a small spike only (Fig. 10). The greater difference observed in this figure between stimuli required to elicit small and large spikes occurs probably because of the relatively refractory period of the "B" area following the large spike.

Excitability of Ventral Root Fibers Following Small and Large Soma Spikes.—The proposed assumption that the small spike results from activity which does not spread over the whole motoneuron has no implications concerning its possible propagation along the axon. Information on this question would, however, be expected to provide useful indications of the location of the region involved in production of the small spike. In order to establish whether or not small spikes resulting from direct stimulation propagate to the axon, the strength of ventral root stimuli required to excite the axon of an impaled motoneuron was measured at various times after that motoneuron was made to fire a small or a full spike. Small or large spikes were elicited by the method illustrated in Fig. 4, and it was found that identical changes occur in the axon in the two cases. This shows that the axon undergoes refractoriness of identical time course following discharge of a small or a full spike from the motoneuron soma. Therefore it must be concluded that following discharge of a small spike propagation along the axon occurs.[2]

Observations on Orthodromic Spikes.—Spikes evoked by orthodromic stimulation present only a barely appreciable inflection in their rising phase and block at this point is more difficult to evoke than it is for spikes elicited by antidromic or by direct stimuli. Partial block was observed, however, to occur at a critical stage of deterioration as an irreversible event (Frank and Fuortes, 1955 *b*). In later experiments partial block could be reversibly obtained in the absence of clear signs of damage, for instance by applying a hyperpolarizing pulse shortly after initiation of the spike. However, since very strong hyperpolarizing pulses were required to evoke partial block, in the experiments performed the records obtained were obscured by large artifacts and therefore

[2] Measurements performed after completion of this study show that these conclusions on site of initiation of directly evoked spikes apply also to spikes of orthodromic origin. Small spikes of orthodromic origin were obtained by applying a short hyperpolarizing pulse to the motoneuron just after initiation of the impulse. Analysis of ventral root excitability following discharge of the small spike revealed that this small spike elicits a conducted impulse in the axon. It appears therefore, that orthodromic spikes are also initiated in the region of the axon hillock.

were unsuitable for analysis. The features of orthodromic spikes observed in this study resemble those of spikes generated by direct stimulation with long depolarizing pulses or by antidromic stimulation applied on a background of depolarization, since in these conditions the inflection on the rising phase becomes less apparent and block becomes more difficult to evoke (Frank and Fuortes, 1956). The results obtained are therefore consistent with the assumption that orthodromic spikes are initiated and spread over the motoneuron soma in a way similar to that occurring for antidromic spikes if it is accepted that synaptic impingement exerts on motoneurons a depolarizing action which outlasts spike initiation. The number of experiments performed in this study on orthodromic spikes was, however, insufficient for reaching a reliable conclusion on details of their mode of initiation and production.

DISCUSSION

Since the results of the various experiments performed did not contradict the basic hypothesis stated above, that the components of motoneuron spikes are the result of activity of different parts of the motoneuron, it is legitimate to attempt an interpretation of the findings on the basis of a more specific statement of the same fundamental hypothesis.

The following assumptions will now be made:

1. Excitation of the motoneuron soma following antidromic or direct stimulation is initiated in a limited region of the cell membrane and spreads from there to other areas by the usual mechanism of conduction, the active region acting as an electrical stimulus for the inactive regions (see Hodgkin, 1937 *a* and *b*).

2. The resistance of soma membrane and cytoplasm is such that the interior of the soma is very nearly isopotential both during activity and during application of currents through the microelectrode.[3] Large potential drops may exist instead between the inside of the soma and the inside of its axon or dendrites (see Brock *et al.*, 1952 *a*).

Some conclusions can be drawn from these assumptions if it is accepted that the electrode is located in the motoneuron soma (see Frank and Fuortes, 1955 *a*). When only a part of the soma membrane is active, the action potential across that part will be reduced by the inactive area whose depolarization it must support. Local activity of the soma will thus give rise to a spike of reduced height. The spikes recorded by the electrode during such local activity will be only a little smaller than the spike at the active area and very slightly larger than the induced depolarization of the inactive area.

[3] During application of currents through the microelectrode a large potential drop occurs in the cytoplasm immediately surrounding the electrode tip. This drop, however, is limited to a very small volume and therefore does not affect the isopotentiality of the greater part of the soma.

Currents through the microelectrode initiate local firing at a threshold depolarization of about 10 mv. (Coombs, Eccles, and Fatt, 1955; Frank and Fuortes, 1956). This local spike will not spread over the rest of the cell, however, until the cell is depolarized by 30 or 40 mv. Thus it becomes necessary to abandon the view that the properties of the motoneuron membrane are uniform in favor of the view that different regions of the motoneuron have different thresholds.

The size of the spikes resulting from isolated activity of the "A" or "B" regions of the motoneuron indicates that the "A" region, where firing is initiated, is small in comparison with the "B" region. The finding that firing of the "A" region alone produces a normal conducted action potential in the ventral root without producing a full sized spike in the soma suggests that the "A" region is located between the axon and the microelectrode, and therefore presumably in the neighborhood of the axon hillock.

Application of these interpretations to a simplified model in which the motoneuron soma is represented by a sphere permits one to show diagrammatically how an impulse may be initiated by a depolarizing constant current through the microelectrode situated in the center of the sphere. The potential drop generated across the membrane by applied currents will be almost equal in all parts of the soma but will decay exponentially along the axon. If the decay of depolarization occurs in a region peripheral to that where threshold is supposed to decrease, excitation will arise somewhere in the region of the axon hillock (Fig. 11 A).

In the case of antidromic excitation, the potential change ahead of an antidromically conducted impulse will decrement along the axon but will decrement only very little in the soma itself, since large potential drops are not supposed to occur in the cytoplasm. As activity progresses towards the axon hillock, the low resistance and the capacity of the soma will act as a load on the axon spike which consequently will decrease in size. The effect on this reduced impulse on the regions immediately ahead is, however, sufficient to evoke firing of a localized region in the neighborhood of the axon hillock. This region should be approximately the same as that which is first excited by direct stimuli, thus permitting one to explain the similar inflection observed in spikes evoked by direct and antidromic stimulation.

The rapid slope of the initial phase of antidromically evoked spikes would be expected if the axon hillock region is excited by a smooth process of conduction progressing with high safety factor. By contrast, the depolarization responsible for initiation of firing following synaptic impingement develops in many conditions with slow time course. In these cases, the familiar inflection between synaptic potential and spike will be recorded.

Firing of the axon hillock evokes passive depolarization of the inactive ("B") parts of the soma membrane and the depolarization recorded at this time by

an inserted microelectrode will be somewhat smaller than the height of the local spike at the axon hillock and just a little greater than the depolarization of the major portions of the soma membrane. Since the threshold of most of the soma is high, this passive depolarization will not necessarily be sufficient to evoke firing of the rest of the soma at once.

If spread of the local impulse is aided by sustained depolarization of the soma (as happens during applied depolarization and probably following synaptic stimulation), firing of the whole soma will occur without delay and little or no inflection will be seen in the rising phase. In other conditions, however, the local impulse may grow only gradually by evoking firing of immediately neighboring areas which are slightly more depolarized and perhaps also possess lower threshold than the distant regions. While this gradual process occurs, the potential recorded by a microelectrode will change slowly and the slope of the rising phase of the spike will decrease. As the area involved by the impulse grows, the inactive area of the soma will decrease, until a point will be reached at which the stimulus supplied by the local activity will be safely sufficient to excite the whole soma. At this point the rise of the recorded spike will again be rapid, progressing in explosive fashion to its peak.

If the threshold of the soma is increased by refractoriness or by hyperpolarization, the local impulse initiated in the "A" region either by antidromic conduction or by direct stimulation may die out before invading the whole soma membrane. In this case a small spike only will be recorded by the microelectrode. When the local activity is just insufficient to evoke firing of a refractory or hyperpolarized soma, it should still be capable of propagating along the axon because the axon has lower threshold and because conduction towards the periphery is not endangered by the increase of area which presumably lowers the safety factor of conduction towards the soma. If a small spike is initiated by antidromic stimulation it could not be expected to propagate back into the axon, since this would be absolutely refractory. If instead the small spike is elicited by direct stimulation when both soma and axon are only relatively refractory or when hyperpolarizing current is delivered through the microelectrode, propagation along the axon occurs, as seen above (p. 744).

Other results of this study may be justified under the proposed assumption that the transition between high and low threshold areas is not abrupt, but occurs gradually over a finite region of the motoneuron membrane as diagrammatically shown in Fig. 11 A. This assumption implies that strong stimuli will excite areas larger than those excited by weak stimuli. During the refractoriness following a full spike, activity of a large area may still be insufficient to elicit generalized firing. Therefore, strong direct stimuli applied in the aftermath of a spike, may elicit large "local" spikes as illustrated in Fig. 2.

Another consequence of the same assumption is that absolute refractoriness may be difficult to detect after local firing since, even if a part of the "A" area is absolutely refractory a direct stimulus may still evoke firing of its remaining

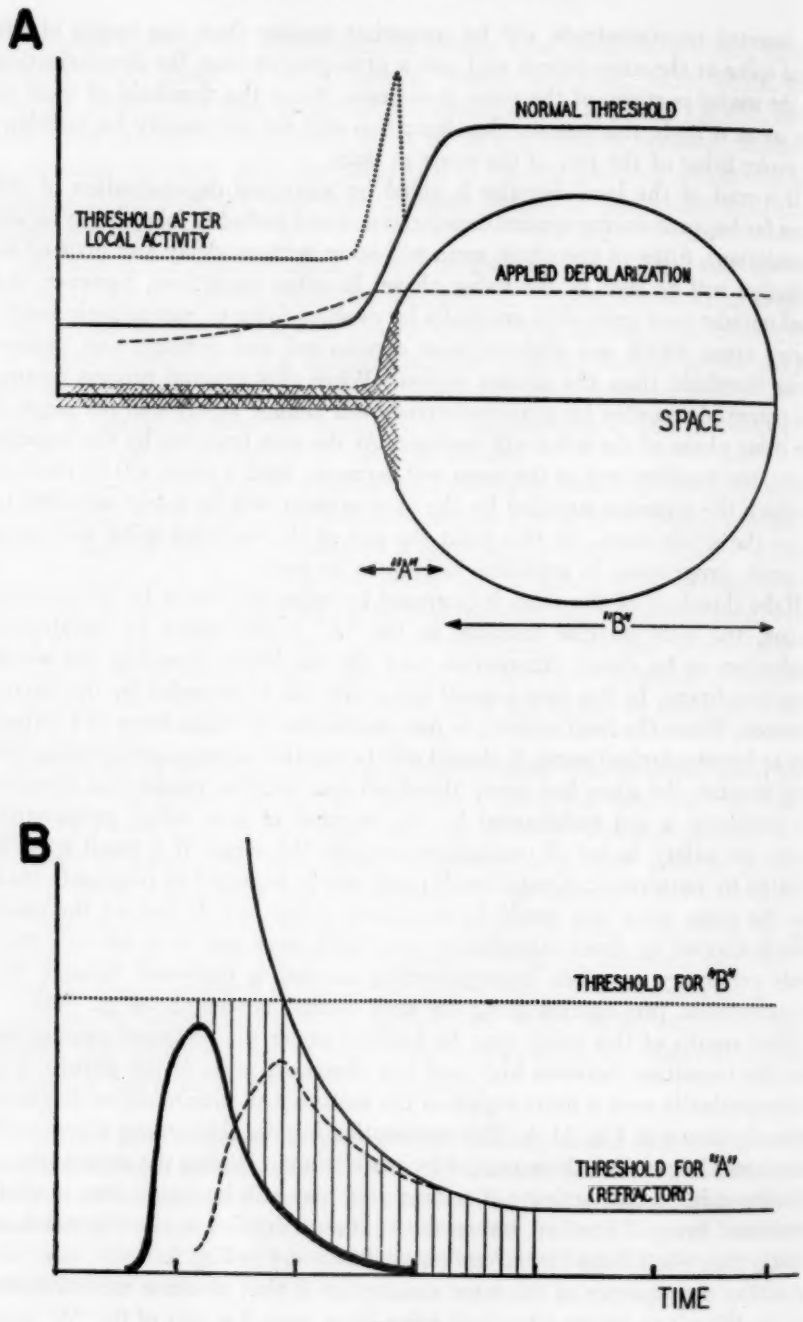
A
NORMAL THRESHOLD
THRESHOLD AFTER
LOCAL ACTIVITY
APPLIED DEPOLARIZATION
SPACE
"A"
"B"
B
THRESHOLD FOR "B"
THRESHOLD FOR "A"
(REFRACTORY)
TIME

FIG. 11

portions before reaching the strength necessary to elicit generalized firing (Fig. 11 A). Therefore a stimulus applied immediately following local activity may still evoke a local rather than a generalized spike, as seen in Fig. 6 A.

Since local activity may be evoked twice in close succession, isolated firing of the "B" area of the motoneuron could be evoked only during the falling phase of the small spike. Thus the potential at the peak of the "B" spike evoked in this way is determined both by activity of the "B" area and by the decaying potential of the small "A" spike. A lower peak potential might be reached by the spike resulting from activity of the "B" area alone.

The diagram of Fig. 11 B is finally presented to explain the changes in excitability and spike form obtained following stimuli producing a small spike only (*cf.* Figs. 5 to 7). It is supposed in the diagram that the height of the small spike was just insufficient to elicit generalized firing and that excitability of the "A" area (as determined by current measurement) followed a curve such as illustrated.

According to this diagram, as stimulus interval is decreased, threshold membrane potential of the "A" area will be at first lower (more negative) later higher than threshold for "B." The strength of the direct stimuli required to elicit firing of the "B" area will steadily decrease when these are superimposed on the small spike since the major portions of the soma membrane will be sub-

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FIG. 11. Diagrams to illustrate initiation of impulses in motoneuron soma.

A. A spherical motoneuron soma is indicated and its axis passing through the center of the axon is used as abscissa of the plot. Ordinate, membrane depolarization measured from resting membrane potential. The dashed (– – –) line indicates the distribution of the potential changes evoked in various portions of the motoneuron by currents applied through a microelectrode situated in the center of the sphere. The solid line represents the depolarization level required to evoke firing of different parts of the motoneuron membrane. In normal conditions threshold stimuli applied through the microelectrode will initiate an impulse in a region where the dashed line is above the solid line.

The dotted line (. . . .) is supposed to indicate the depolarization required to elicit firing shortly after activity which has involved only the shaded areas of the motoneuron. A stronger stimulus is required in these conditions to evoke firing, but the soma impulse may still be initiated in the region of threshold transition ("A").

B. In this diagram, ordinate represents soma membrane depolarization as in Fig. 11 A, while abscissa represents time in milliseconds. It is assumed that, following activity of the "A" region of the motoneuron (indicated by the small spike), threshold for excitation of "A" is temporarily increased as shown by the solid line, while threshold for excitation of "B" remains unchanged (dotted line). The vertical lines indicate the height of depolarization required to reach threshold after and during local activity. At short intervals threshold of "B" will be reached before threshold of "A." The dashed curve measures the height of the vertical lines from baseline and resembles the experimental excitability curves illustrated in Figs. 9 and 10.

jected to the depolarization brought about by the sum of the small spike and the directly applied stimuli. The dashed line is a plot of the strength of the stimulus capable of evoking firing as a function of time, constructed on the basis of this diagram. Its resemblance to the excitability curves obtained experimentally is apparent (*cf.* Figs. 9 and 10).

It will be noted that the present hypothesis is perhaps more specifically stated but not essentially different from the hypotheses previously set forth by Araki and Otani (1955) and by Eccles (1956). Also, the results of the present study do not contradict the view expressed by the same authors that orthodromic excitation originates in a region of the motoneuron membrane adjoining the axon hillock, but no satisfactory test of this assumption has been worked out.[4]

Some difference exists instead between these views and another proposed interpretation. Fatt (1957) found signs of centrifugal conduction along the dendrites when a full spike is recorded from the soma but not otherwise. From this he concluded that the initial portion of the spike is due to soma activity whereas the portion above the inflection is due to firing of dendrites. While his experimental results do not contradict the views expressed in the present paper, Fatt's interpretation cannot be reconciled with the observation (Crain, 1956) that spikes elicited by antidromic or direct stimulation of spinal ganglion cells of frogs in tissue culture present the same inflection described above despite absence of dendrites in these cells.

It should be noted that not all details of the experimental results have been adequately considered in relation to the proposed model. This was advisable for the sake of simplicity, since several of the observed phenomena, although not inconsistent with the proposed hypothesis, could be included only with the addition of further assumptions. Conversely, for the sake of clarity, the proposed model was made more specific than was actually required by the results.

For instance it has been assumed (Fig. 11 A) that the "A" area is a continuous region around the axon hillock. Under this assumption antidromic impulses could propagate to the "A" area through a process of continuous conduction and thus the high safety factor for antidromic excitation of the "A" area could readily be accounted for. It is possible, however, that the "A" area consists instead, of small spots scattered over soma and dendrites as might occur, for instance, if the regions under the synaptic knobs had properties different from those of the other regions of the motoneuron membrane.

Also, it has been assumed throughout the foregoing discussion that the potentials recorded by the microelectrode faithfully reflect the changes occurring at any area (including the "A" area) of the soma membrane. This will be the case if (1) only small potential drops occur within the soma cytoplasm, and (2) the microelectrode tip is located in the cytoplasm. If either of these two

[4] However, see footnote 2.

conditions does not apply, then the recorded potential changes may be different from those occurring at the "A" area of the soma, which have been presumed to be critically involved in initiation of excitation (see Frank and Fuortes, 1956, p. 467). For mixed synaptic impingement, the recorded potential change could differ even in sign from the change occurring at the "A" region.

Finally, whereas it is assumed in the present discussion that changes in the size of the recorded spike are due to changes of area of the firing membrane, it is quite possible that at least some of the observed size variations may have a different origin.

Despite these shortcomings, the simplified model proposed in this discussion appears to provide a not unreasonable explanation of the principal findings of the present study.

SUMMARY

1. Spikes evoked in spinal motoneurons by antidromic stimulation normally present an inflection in their rising phase. A similar inflection is present in spikes evoked by direct stimulation with short pulses.

2. In either case the inflection becomes less prominent if the motoneuron membrane is depolarized and more prominent when it is hyperpolarized. Both antidromic and direct spikes may fall from the level of the inflection thus evoking a "small spike" only if sufficient hyperpolarization is applied. Similar events occur when antidromic or direct spikes are evoked in the aftermath of a preceding spike.

3. Spikes evoked by direct stimuli applied shortly after firing of a "small spike" may also become partially blocked at a critical stimulus interval. At shorter intervals, however, spike size again increases and no inflection can be detected in the rising phase.

4. When a weak direct stimulus evokes a small spike only, a stronger stimulus may evoke a full spike. Curves of the strength of the stimuli required for eliciting small or full spikes have been constructed in a number of conditions.

5. To explain the results it is assumed that threshold of the major portions of the soma membrane is higher than the threshold of the axon, the transition occurring over a finite area near the axon hillock. Following antidromic or direct stimulation, soma excitation is then initiated in the region of the axon hillock. Spread of activity towards the soma occurs at first slowly and with low safety factor. At this stage block may be easily evoked. Safety factor for propagation increases rapidly as the growing impulse involves larger and larger areas of the soma membrane so that, once the critical areas are excited, activation of the remaining portions of the soma membrane will suddenly occur.

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THE RELATIONSHIP BETWEEN SODIUM, POTASSIUM, AND CHLORIDE IN AMPHIBIAN MUSCLE*

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(Received for publication, December 19, 1956)

In a previous publication (28) it was shown that the cells of the sartorius muscle of the toad *Bufo marinus* were able to maintain their internal K^+ at an absolute level despite great fluctuations (0 to 25 m.eq. per liter) of the external K^+. When the external level was raised to 50 m.eq. per liter there was an intracellular increase amounting to about 17 per cent of the control level in normal Ringer. This is in contrast to the capacity of the cell to maintain an absolute ratio Na^+_{out}/Na^+_{in} of approximately 3:1 in the face of large variations in the external Na^+ (45 to 650 m.eq. per liter).

Boyle and Conway (2) had shown that K^+ entered the cell in proportion to the increase in external K^+, from which they drew the conclusion that the intracellular K^+ content would result from the electrochemical gradient, the product $K^+_{in} \times Cl^-_{in}$, being equal to the product $K^+_{out} \times Cl^-_{out}$.

In this paper it is our intention to correlate the changes in the intracellular Na^+ and K^+ content brought about by alterations in the external ionic environment with the concomitant movements in chloride ion.

A bibliography of the earlier literature relating to the estimation of chloride in muscle has been given by Fenn (9). We shall refer later to the work of Carey and Conway (3), Levi and Ussing (18), and Harris and Martins-Ferreira (10).

The method of estimation of chloride employed by some of the earlier workers (the Van Slyke acid digest), has been shown by Heilbrunn and Hamilton (11) and Wilde (34) to yield results up to 30 per cent too low. The methods used by us for the estimation of Na^+, K^+, and Cl^- were such that all three ions could be estimated on the same muscle. This has not been possible with previous methods.

The movements of Na^+ and Cl^- are so closely linked that we have formed a tentative hypothesis that these ions exist together in an intracellular phase. The amounts of the two ions in this phase are usually equivalent, and depend on the concentration of the same ion in the external medium. The amount of

*This work was undertaken with the aid of a grant from the National Health and Medical Research Council of Australia.

intracellular chloride is little, if at all, influenced by the K^+ content of the cell or by movements of this ion. The results will be employed (*a*) to show that the accumulation of intracellular K^+ is not associated with a Donnan mechanism, and (*b*) to suggest that the existence of a sodium pump in the resting muscle cell is an unnecessary postulate.

Methods

Treatment of Muscles.—Muscles which were analysed *in vivo* were carefully dissected from the animal, blotted, weighed, and immediately subjected to the analytical procedure outlined below. Isolated muscles were removed from the animal, blotted and weighed, soaked in the appropriate solution for the stated time, blotted, weighed again, and then analysed. It will be noted that we did not obtain a dry weight for our muscles, as in previous experiments. This was due to the fact that the analysis of chloride required a wet extraction. The results of our analyses over the preceding 4 years had shown that the water content of muscles *in vivo* was constant (27). By means of the above two weighings any volume change could be detected, and hence any change in dry weight.

Usually 10 pairs of muscles were used in each experiment. The companion muscle always served as a control for the treated, and unless otherwise stated was soaked in normal Ringer for a comparable time. The results were then subjected to the appropriate statistical analysis.

Collection of Blood.—Blood was taken by cardiac puncture into a heparinized syringe. It was placed in a centrifuge tube under an oil seal, and spun immediately.

The Estimation of Na^+, K^+ and Cl^-.—Preliminary experiments had shown that there was an almost complete loss of Cl^- on ashing at 560°C. Eventually it was found that the following technique was satisfactory. The muscle was very finely sliced in a centrifuge tube with scissors, the scissors were then thoroughly washed down with N/50 nitric acid, and the volume made up to 10 ml. in a centrifuge tube. The tubes were allowed to stand at room temperature for 48 hours. They were then stirred thoroughly and centrifuged; 3 ml. of the supernatant was accurately removed for the estimation of Na^+ and K^+ by means of a Beckman flame spectrophotometer. The proteins in the remainder of the supernatant were precipitated by the method of Somogyi (31), and the filtrate was used for the estimation of Cl^-. The efficiency of the extraction method was compared with ashing techniques on companion muscles, and the results showed that the removal of Na^+ and K^+ was complete.

The chloride ion was estimated by a potentiometric technique similar to that of Sanderson (22). We are indebted to Mr. Gordon Bennett for the following modification of the above method.

The titration apparatus consisted of an inert reference electrode of platinum mounted in the tip of a microburette containing 0.005 N silver nitrate. The burette tip was just immersed in the chloride solution, which was contained in a small glass bucket. A pure silver wire electrode was placed in the bucket, and the contents were stirred by a constant speed electric motor.

The main functional modifications of Sanderson's circuit were such as to achieve the greater sensitivity and higher stability necessary when working with chloride

solutions of the order of 1,000-fold more dilute than this author used. Stability was achieved by operating all electrical apparatus from a voltage regulator transformer, and the use of voltage regulator tubes in the rectifier circuit. These tubes also supplied reference voltages which were used to ensure reproducible zero and full scale deflections of the indicating meter. The system had a high input resistance, and a linear response to input voltage, consequently a microammeter with an arbitrary linear scale was used as indicator of the electromotive force across the titration cell.

The standard $AgNO_3$ made up for the titrimeter was checked daily with the standard NaCl solution made up for the flame spectrophotometer, and the titration agreed within 1 per cent of the calculated normality. The NaCl solution was regarded as the absolute standard, so that the error in determining Na^+ and Cl^- in a muscle should be confined to the error inherent in the methods.

Extracellular Space.—The extracellular space was determined by the method of Wilde (34). The mean value of 8 determinations was (±S.E.), 15.8 ± 0.6. Some values must be as low as about 10.8 per cent, assuming there is no intracellular Na^+ (see reference 28 for discussion). These values are lower than those found by Harris and Martins-Ferreira (10) in frog muscle soaked in Ringer. These latter findings are based on the assumption that there is no intracellular chloride, an assumption with which we cannot agree (see below).

Solutions.—Ringer solutions used have been described (24), and were buffered with phosphate or bicarbonate. In low Na^+ Ringer the osmotic pressure was made up with sucrose.

RESULTS

In Vivo Muscles.—In order to obtain the intracellular ionic content of the muscles in the living animal it was necessary to determine the level of these ions in the plasma. The plasma was taken from each animal, and analysed separately. The mean ionic content ±S.E. of the plasma was Na^+ 122 ± 1.7 m.eq. per liter, K^+ 2.7 ± 0.02 m.eq. per liter, Cl^- 91.7 ± 2.4 m.eq. per liter (31 observations).

The correlation between Na^+ and Cl^- in the sartorius muscle is shown in Fig. 1. The complete lack of any relationship between K^+ and Cl^-, and Na^+ and K^+, is shown in Figs. 2 and 3. In an earlier publication (27) we noted a marked variation in the Na^+ content of sartorius *in vivo*. In the particular series analysed for this paper the variation was not so definite, most muscles having a low Na^+ (and Cl^-) content. We were, however, able, following the technique of Fenn *et al.* (8), to "create" high Na^+ toads by permitting the animal to bathe in 0.8 per cent NaCl solution for 2 to 3 days. To prevent contamination, the solution was changed twice daily. The increase in plasma Na^+ and Cl^- brought about by this procedure is small compared with the large increment in the muscle.

The correlation between Na^+ and Cl^- is highly significant, and the relationship has been analysed as follows:—

A multiple regression of Na^+ and K^+ as determinants of Cl^- was computed.

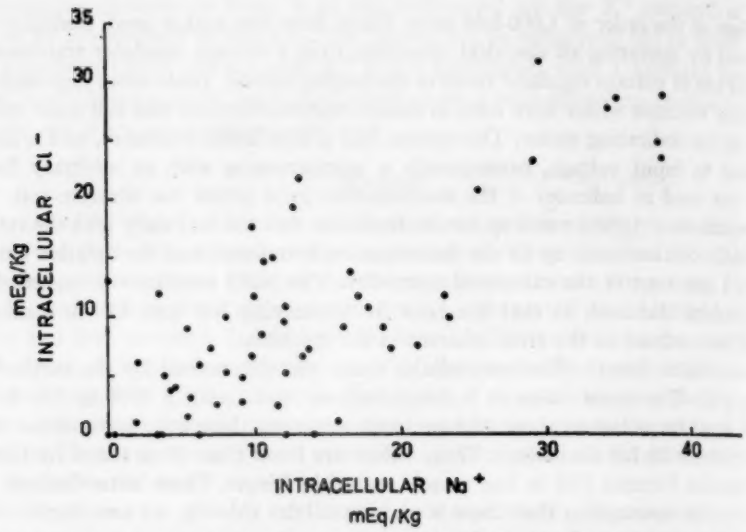


FIG. 1. The relationship between intracellular Na^+ and Cl^- in the intact animal. In this and subsequent figures each point represents the analysis of one muscle. For regression coefficient see text.

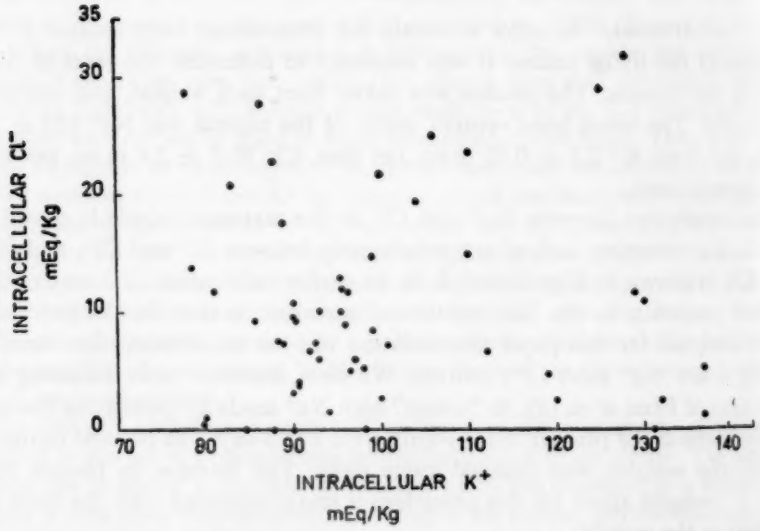


FIG. 2. The relationship between intracellular K^+ and Cl^- in the intact animal. There is no significant correlation.

Partial regression coefficients and their standard errors were obtained as follows:—

Na^+, $b_1 = 0.671$ S.E. $= 0.072$, significant at 1 per cent level.

K^+, $b_2 = 0.067$ S.E. $= 0.045$, not significant.

On recalculating the regression ignoring K^+, the coefficient was significant at the 1 per cent level, and the equation was obtained:

$$Cl^- = 1.22 + 0.658\ Na.^+$$

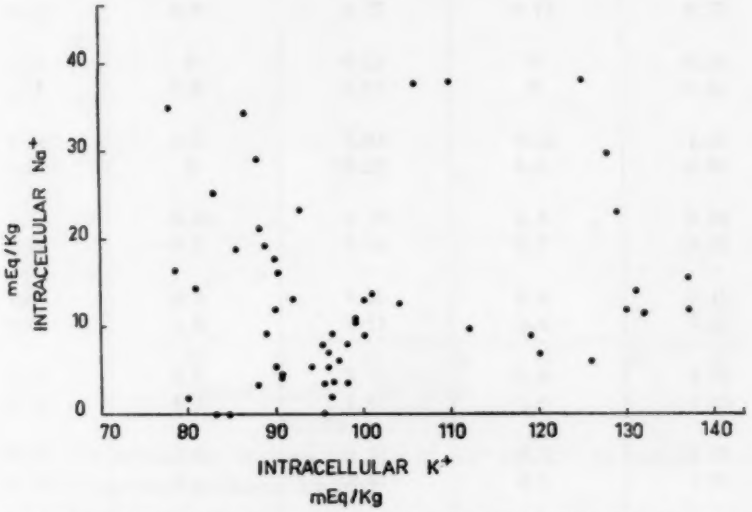


FIG. 3. The relationship between intracellular Na^+ and K^+ in the intact animal. There is no significant correlation.

This was compared with the slope 92/122 (0.754), being the ratio of Cl^- to Na^+ in plasma.

$$t = \frac{0.754 - 0.671}{0.072} = 1.16, \text{ not significant.}$$

Hence the experiment does not constitute evidence against 0.754 as slope.

The relation between Na^+ and Cl^- (Table I) within paired muscles was investigated. The regression of Cl^- on Na^+ was significant at the 5 per cent level.

Muscles Soaked in Normal Ringer (Isolated Muscles).—The ionic content of muscles *in vivo* was compared with that of the companion muscle soaked for 4 hours in normal Ringer. It is well known that when muscles are soaked in normal Ringer there is a gain of Na^+ and loss of K^+, until at the end of 2 hours a

TABLE I

Ionic Content of Paired Muscles in Vivo

Total Na, *m.eq./kg. wet weight*	Intracellular Na, *m.eq./kg. wet weight*	Total Cl, *m.eq./kg. wet weight*	Intracellular Cl, *m.eq./kg. wet weight*	Intracellular K, *m.eq./kg. wet weight*
22.2	5.4	20.9	9.0	96.0
20.7	3.5	23.6	12.0	95.6
32.3	17.0	23.2	11.8	81.0
22.2	5.4	14.7	1.6	94.0
19.1	1.9	14.3	1.0	86.0
27.9	12.0	21.4	9.6	90.0
13.4	0	12.9	0	84.6
14.5	0	13.5	0.5	79.8
36.1	21.0	19.7	7.6	88.3
20.4	3.4	12.0	0	87.8
24.4	8.0	21.8	10.0	95.4
23.4	7.0	19.5	7.4	92.6
21.6	4.5	16.9	4.0	90.6
22.7	5.6	15.8	3.1	90.0
24.3	8.0	17.8	5.2	98.0
23.1	6.2	18.1	5.9	97.0
20.8	3.8	17.5	5.0	96.5
19.7	2.0	18.3	6.0	93.0
32.4	9.8	28.3	17.6	89.0
32.4	9.8	23.2	11.7	96.3
21.2	4.2	16.4	3.8	90.6
17.6	0	24.5	13.0	95.4
20.9	3.6	9.2	0	98.0
25.4	9.0	15.5	2.6	100

In this and subsequent tables ionic contents are calculated on a wet weight basis and refer to intracellular levels, assuming 15 per cent extracellular space.

There is an apparent large variation in the intracellular Na^+ and Cl^- content of paired muscles. This variation is probably due to slight differences in the extracellular space in individual muscles. When the total content of an ion is contained mainly in the extracellular space (*e.g.*, Na^+ and Cl^-) slight variations in this space produce large differences in the estimated intracellular content. This disadvantage does not occur when the concentration of the constituent in the external fluid is small (*e.g.*, K^+). Thus in the above table the paired analyses agree better for K^+ than they do for Na^+ or Cl^-.

new steady state is established. It has also been known for some time (7, 8, 21) that the total Cl^- content of muscles increases on soaking in Ringer. Some of these authors (7, 8) were loath to admit that the Cl^- had passed into the cell. Boyle and Conway (2) followed the rate of entry of Na^+ and Cl^- into the cell and the concomitant K^+ loss. They found an approximately equivalent Na^+ gain and K^+ loss, and the anion gain could be considered equivalent to either the Na^+ gain or the K^+ loss. These authors also noted that the ratio K^+_{in}/K^+_{out} was not equal to Cl^-_{out}/Cl^-_{in} in muscles soaked in normal Ringer, and ascribed this to a drop in resting potential. It has since been shown (27) that such a fall in potential does not occur.

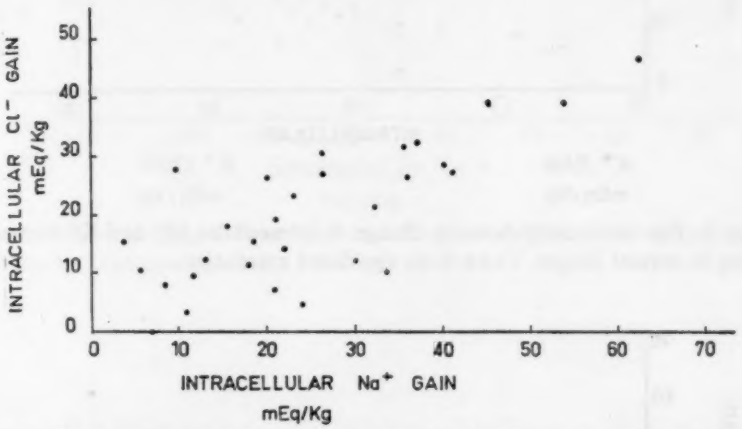


FIG. 4. The relationship between the gain of Na^+ and Cl^- on soaking in normal Ringer. For regression coefficient see text.

In Fig. 4 gain in Na^+ has been plotted against gain in Cl^-. In this particular series 7 out of 30 muscles did not lose K^+ on soaking. This is a rather unusual occurrence, but it enabled us to show that the correlated gain of Na^+ and Cl^- was independent of any K^+ movement. The correlation between the gains of Na^+ and Cl^- was highly significant ($r = 0.80$). Fig. 4 is drawn from the results obtained with 30 pairs of muscles. Figs. 5 and 6 illustrate the lack of relationship between gain of Cl^- and loss of K^+ and between loss of K^+ and gain of Na^+.

In addition to the results presented in Figs. 4, 5, and 6, which were obtained by a comparison of paired muscles, one *in vivo*, the other soaked in normal Ringer, we were able to correlate the ionic content in a very large series (124 muscles) of soaked muscles. These results have been culled from other experiments in which muscles soaked in normal Ringer served as controls. There are, of course, no *in vivo* mates for these muscles, since their companions were subjected to other experimental conditions. Hence in Figs. 7, 8, and 9 the absolute amounts of the ions are plotted, and not the gains or losses.

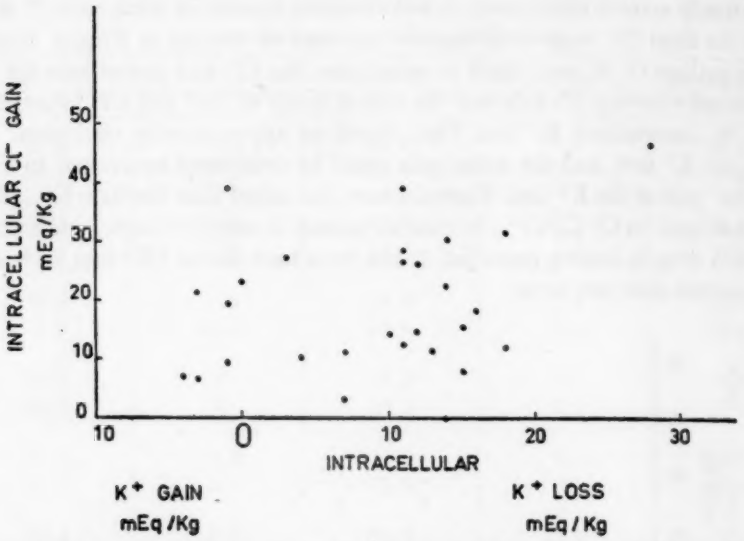


FIG. 5. The relationship between change in intracellular Cl^- and K^+ content on soaking in normal Ringer. There is no significant correlation.

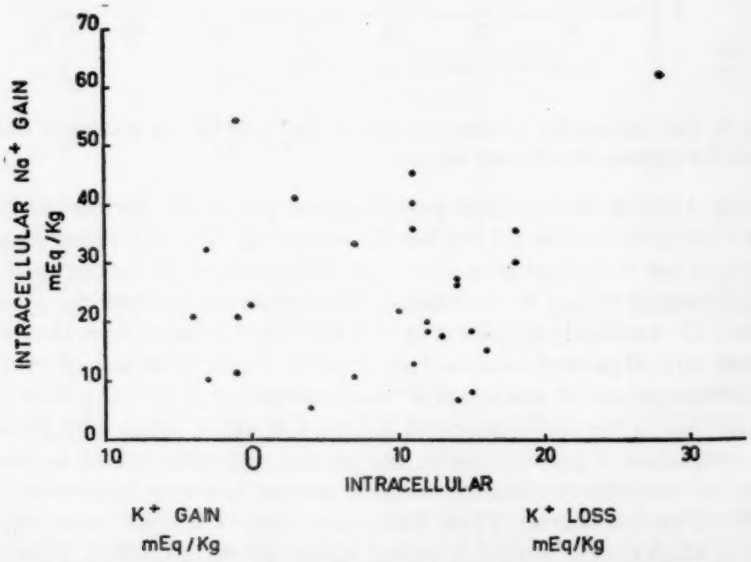


FIG. 6. The relationship between change in intracellular Na^+ and K^+ content on soaking in normal Ringer. There is no significant correlation.

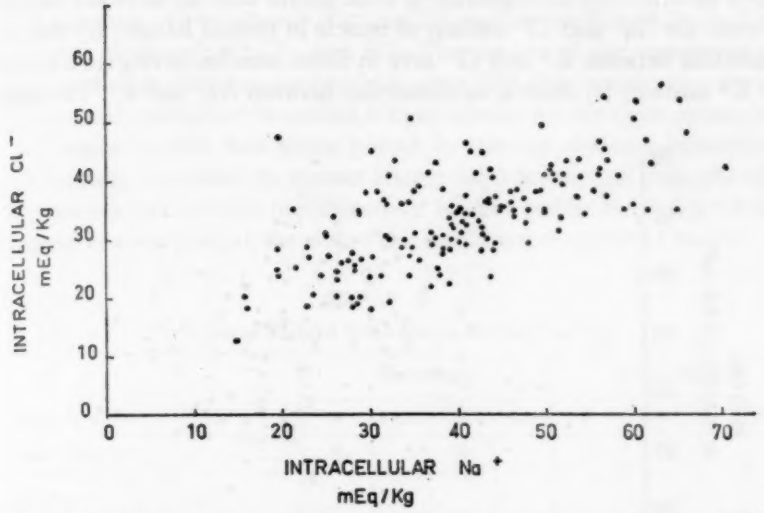


FIG. 7. The relationship between Na^+ and Cl^- in sartorii soaked in normal Ringer For regression coefficient see text.

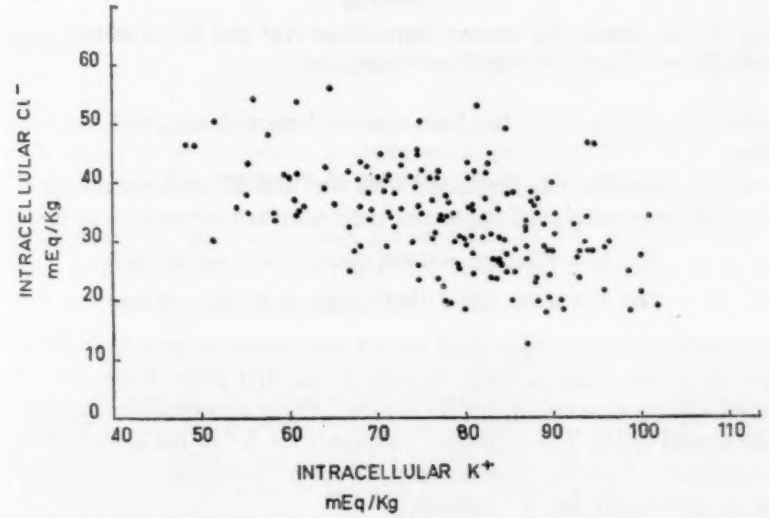


FIG. 8. The relationship between intracellular K^+ and Cl^- in sartorii soaked in normal Ringer. For discussion of correlation see text.

It is obvious from an inspection of these graphs that (*a*) there is a correlation between the Na^+ and Cl^- content of muscle in normal Ringer; (*b*) there is no connection between K^+ and Cl^- save in those muscles having an abnormally low K^+ content; (*c*) there is no connection between Na^+ and K^+. The relation-

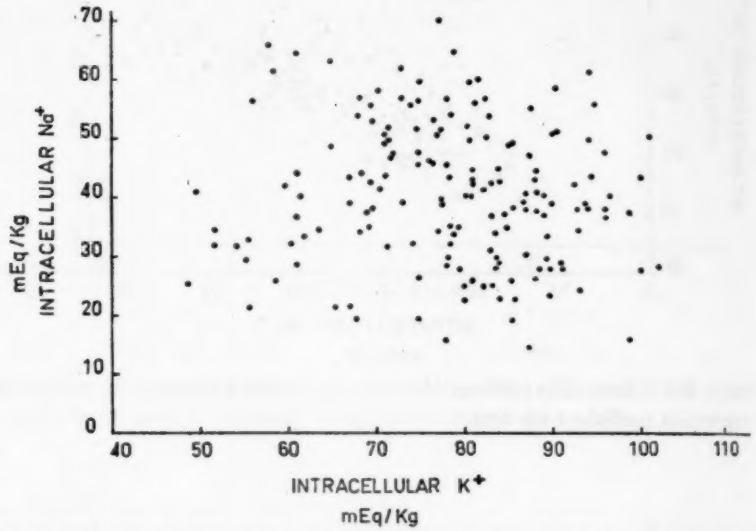


FIG. 9. The relationship between intracellular Na^+ and K^+ in sartorii soaked in normal Ringer. There is no significant correlation.

ship between Na^+ and Cl^- has been examined more closely, with the following results:

A multiple regression was performed with Na^+ and K^+ as determinants of Cl^-. The partial regression coefficients and their standard errors were as follows:—

Na^+, $b_1 = 0.582$, S.E. $= 0.050$, significant at 1 per cent level.

K^+, $b_2 = 0.196$, S.E. $= 0.065$, significant at 1 per cent level.

The experiments from which these figures were obtained were divided into two halves on a chronological basis, because in the first series there were several muscles with an abnormally low K^+ content. These abnormalities did not occur in the second series. The regression coefficient for K^+ in the second series was not significant. Thus the dependence of Cl^- on K^+ is only seen in muscles which have an abnormally low K^+ content.

When the regression coefficient for Na^+ is compared with the ratio Cl^-/Na^+ in Ringer, *i.e.* 110/130 (0.846), it is found that the difference is highly significant ($t = 5.31$, 123 d.f.). Thus the ratio of Cl^-/Na^+ in the isolated muscle differs from that of the surrounding Ringer.

The Effect of Alteration of External NaCl Level.—We have investigated the effect of alteration of the external Na^+ and Cl^- level of the Ringer on the intracellular ionic content ($Na,^+$ Cl^-, and K^+). The pattern of the experiment was the same as that described in our previous paper (28). One muscle was removed from the animal and soaked in normal Ringer solution for the appropriate time. The companion muscle was either placed in the test solution immediately (unequilibrated), or soaked in normal Ringer for 2 hours, and then placed in the appropriate test solution (equilibrated). In the equilibrated series the control muscle was analysed at the end of the equilibration period (2 hours).

TABLE II

The Effect of Reduced NaCl Ringer on Ionic Content

	Equilibrated				Not equilibrated			
Na in Ringer, *m.eq./liter*	65	Ratio	56	Ratio	65	Ratio	45	Ratio
Cl in Ringer, *m.eq./liter*	55		37		55		37	
Na, *m.eq./kg.*								
Control	40.2	3.2	31.9	4.1	39.1	3.3	32.1	4.2
Treated	29.6	2.2	23.2	2.4	17.6	3.7	20.7	2.2
Cl, *m.eq./kg.*								
Control	34.7	3.2	35.4	3.1	34.1	3.2	32.4	3.4
Treated	25.5	2.2	14.0	2.6	17.3	3.2	18.2	2.0
K, *m.eq./kg.*								
Control	72.8		67.2		83.2		85.6	
Treated	69.7		72.0		81.0		86.2	

"Ratio" means Na^+ or Cl^- (out/in). Control muscles were soaked in normal Ringer for 2 or 4 hours (see text). The changes in K^+ concentrations are not significant.

The external Na^+ level was varied between 45 and 460 m.eq. per liter, the Cl^- level between 37 and 440 m.eq. per liter. In low Na^+ solutions the osmotic pressure was made up with sucrose. High NaCl solutions were of necessity hypertonic.

Low NaCl Ringer (Unequilibrated).—As will be seen from Table II muscles placed in reduced NaCl Ringer lose intracellular Na^+ and Cl^- in proportion to the decrease in the NaCl level of the external solution. Thus, after 4 hours' soaking, when the muscle has reached a steady state the ratios Na^+_{out}/Na^+_{in} and Cl^-_{out}/Cl^-_{in} are the same in the half NaCl Ringer as in the controls. In the one-third NaCl Ringer the ratios are smaller in each case, but the Na^+ and Cl^- ratios are equal. There was no concomitant change in the K^+ level of the muscle in either case.

Low NaCl Ringer (Equilibrated).—When a muscle is soaked in Ringer it

tends to gain NaCl until a steady state is achieved, when the ratio of Na^+ and Cl^- (out/in) is approximately equal to three (see previous section). If the muscle is now removed to a NaCl-deficient solution these ions are extruded from the cell, against a concentration gradient, until the Na^+ and Cl^- ratio is almost completely reestablished. These results are set out in Table II. As was found in the unequilibrated series there was no change in K^+ levels. The relation of this Na^+ extrusion to the Steinbach (32) "pump" was discussed previously (26). This work has recently been confirmed by Van der Kloot (17).

The above results show that there is an extrusion of Cl^- against a concentration gradient. Since the levels of Na^+ and Cl^- are different in the external solution (*viz.* 130 and 110 m.eq. per liter), it is better to consider changes in

TABLE III

The Effect of High NaCl Ringer on Ionic Content

Na in Ringer, *m.eq./liter*	240	Ratio	460	Ratio
Cl in Ringer, *m.eq./liter*	220		440	
Volume change, *per cent*	−13.8		−17.2	
Na, *m.eq./kg.*				
Control	36.0	3.6	39.1	3.3
Treated	93.6	2.6	248	1.85
Cl, *m.eq.k/g.*				
Control	34.6	3.2	34.1	3.2
Treated	87.2	2.5	249	1.8
K, *m.eq./kg.*				
Control	82.0		83.2	
Treated	88.0		83.2	

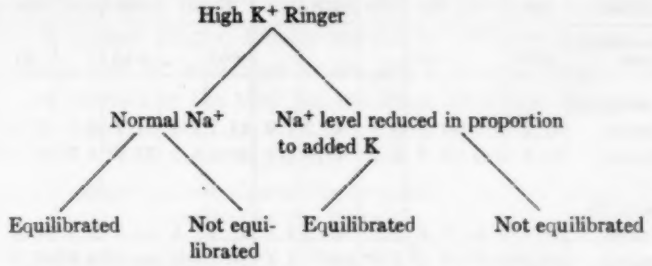
ratio rather than the absolute amount of material moved. It will be seen from Table II that the Na^+ and Cl^- ratios in the treated muscles are nearly identical, thus the extrusion of both ions is of a similar magnitude. The question whether it is necessary to postulate a Cl^- "pump", or whether an alternative mechanism must now be sought is referred to in the Discussion. These results are unique, since for the first time there has been demonstrated a gross movement of the Cl^- ion which is completely unaccompanied by a movement of K^+.

High NaCl Ringer (Unequilibrated).—The results of soaking muscle in high NaCl Ringer are set out in Table III. In agreement with our earlier results (28), it was found that as the osmotic pressure of the external solution is increased there is a progressive shrinkage of the muscle which amounts to approximately 20 per cent at 400 per cent NaCl. This shrinkage reaches a maximum after the first 30 to 45 minutes. The Na^+ and Cl^- have entered the muscle

in proportion to the external increase, but contrary to our earlier results (28) the ratio Na^+ and Cl^- (out/in) has declined at the higher level of NaCl; the Na^+ and Cl^- ratios are, however, still equal.

The Effect of Alteration of the External KCl Level

The plan of our experiments is set out in the diagram below:



The experiments were divided into two series. In one series KCl was added to normal Ringer. In the other series as the K^+ was increased an equivalent amount of Na^+ was subtracted, and thus the Cl^- level remained constant. As in the previous section (the effect of the alteration of external NaCl level) the muscles were either placed directly in the high K^+ solution (unequilibrated), or permitted to equilibrate in Ringer for 2 hours before they were placed in the high K^+ solution. Muscles immersed in normal Ringer gain Na^+ and Cl^- and frequently lose K^+ during the first 2 hours. If these changes are not reversed by raising the K^+ in the Ringer this will produce differences between the equilibrated and unequilibrated series.

The results of our experiments are set out in Tables IV and V. It will be seen that in K-free Ringer there is no significant difference in ionic content between the treated muscle and the control muscle soaked in normal Ringer. It must be remembered that the control muscles have gained Na^+ and Cl^-, and have probably lost K^+, and so these movements have not been affected by soaking in the K-free solution. It will be observed that the cell contained 27 m.eq. per kg. Cl^-, thus it cannot be said that there was insufficient Cl^- to accompany the K^+ from the cell (see Discussion).

In our previous paper (28) we have found that increasing the KCl level to 25 and 50 m.eq. per liter produced a slight increase in the K^+ content of the cells. This increase was by no means equal to that predicted by the Donnan theory. The results set out in Tables IV and V are in agreement with this earlier work. There is no difference in K^+ increase between the series in which KCl was added to normal Ringer and that in which an equivalent amount of NaCl was subtracted. However, the muscles in the latter series increased in volume and showed an increase in Na^+ gradient in high K^+ Ringer. There is little difference between the equilibrated and unequilibrated series, the muscles in the

TABLE IV

The Effect of High KCl Ringer on Ionic Content
Na^+ Level of Ringer Not Altered

	Equilibrated						Unequilibrated						
K in Ringer, *m.eq./liter*	25	Ratio	50	Ratio	87	Ratio	0	Ratio	25	Ratio	50	Ratio	103	Ratio
Cl in Ringer, *m.eq./liter*	135		161		217		107		135		161		223	
Volume change, *per cent*	+2.3		−4.4				+0.5		+3.1		±0		−3.0	
Na, *m.eq./kg.*														
Control	42.2	3.1	41.5	3.1	45.2	2.9	43.7	3.0	31.7	4.1	41.6	3.1	63.7	2.0
Treated	42.8	3.0	38.7	3.4	47.9	2.7	40.6	3.2	22.9*	5.7*	38.0*	3.4*	58.1*	2.7*
Cl, *m.eq./kg.*														
Control	35.4	3.4	31.5	3.5	40.3	3.3	32.3	3.4	33.6	3.3	40.8	2.7	38.8	2.8
Treated	49.6*	2.9*	55.4*	2.9*	100*	2.2*	27.7	3.9	43.5*	3.1*	65.3*	2.5	92.9*	2.4*
K, *m.eq./kg.*														
Control	69.7		63.3		80.6		80.9		64.6		60.5		87.5	
Treated	79.7*		83.3*		142*		77.8		78.8*		94.4*		149*	

* Changes are significantly different from the control ($P < 0.05$).

TABLE V

The Effect of High K^+ Ringer on Ionic Content
Na^+ Level of Ringer Reduced Proportionately

	Equilibrated				Unequilibrated			
K in Ringer, *m.eq./liter*	25	Ratio	50	Ratio	25	Ratio	50	Ratio
Cl in Ringer, *m.eq./liter*	110		110		110		110	
Volume change, *per cent*	+3.1		+15.0		+7.2		+15.4	
Na, *m.eq./kg.*								
Control	31.9	4.1	36.5	3.5	57.9	2.2	42.9	2.8
Treated	16.4*	6.4*	11.4*	7.0*	43.3*	2.4*	18.1*	5.3*
Cl^-, *m.eq./kg.*								
Control	27.4	4.0	31.2	3.5	40.8	2.7	35.2	3.1
Treated	26.8	4.1	36.0	3.1*	46.2	2.4	40.9	2.7
K, *m.eq./kg.*								
Control	80.6		89.2		75.3		82.7	
Treated	89.7*		105*		89.2*		103*	

* Changes are significantly different from the control ($P < 0.05$).

latter contain more K^+ in high K Ringer than the former. Thus it would appear that the loss of K^+ found on soaking in normal Ringer has been hindered in the unequilibrated series, and that in the equilibrated series there has been a smaller return of K^+ to the cell than was found in the former experiments.

Throughout the series of experiments with high K^+ we have used as our control the K^+ content of the companion muscles soaked in normal Ringer. Alternatively one could have used the K^+ value *in vivo*, which would normally be higher than that in normal Ringer. Ideally one should compare the results of the muscles soaked in high K^+ with those *in vivo* and in normal Ringer. Unfortunately this is not possible as the toad has not three hind legs. We have endeavoured to overcome this difficulty in the following manner. Muscles from 12 toads were dissected on the one day, 6 were analysed immediately, and their companions soaked in normal Ringer to which had been added 50 m.eq. per liter KCl. Six were soaked in normal Ringer and their companions soaked in the high KCl Ringer. The results are given in Table VI. The figures show that the high KCl Ringer has caused an entry of K^+ into the cell, because the value is 15.1 m.eq. per kg. higher than that of the muscles *in vivo*. On the other hand the muscles soaked in normal Ringer lost 17.4 m.eq. per kg. K^+, compared with the *in vivo* group. When the companion muscles were soaked in high K Ringer this loss was prevented, and a further 18.5 m.eq. per kg. K^+ entered. This latter addition is in reasonable agreement with the 15.1 m.eq. per kg. K^+ which entered in the first group.

TABLE VI

The Effect of High KCl Ringer on Ionic Content Compared with Controls in Normal Ringer and in Vivo (See Text)

	Control in normal Ringer		Control *in vivo*	
		Ratio		Ratio
Na, *m.eq./kg.*				
Control	53.0	2.5	2.1	57.0
Treated	50.0	2.6	36.2*	3.6*
Cl, *m.eq./kg.*				
Control	38.2	2.9	6.2	18.0
Treated	69.1*	2.3	64.2*	2.5*
K, *m.eq./kg.*				
Control	66.5		83.9	
Treated	102*		99.0*	

Intracellular contents *in vivo* are calculated using plasma ionic levels. The test Ringer contained 50 m.eq. KCl added to normal Ringer.

* Changes are significantly different from controls ($P < 0.05$).

In the high KCl Ringer series (Na^+ level remaining constant) there has been no change in the internal Na^+ level in the equilibrated series. Muscles without equilibration showed a slight decrease in Na^+ content. When the Na^+ level of the Ringer was decreased in proportion to the added K^+ there was a marked decrease in Na^+ content, which was greater than would have been expected from the decrease in Na^+ level of the Ringer. This has resulted in an increase in the ratio Na^+_{out}/Na^+_{in}.

When we come to consider the alteration in internal Cl^- resulting from the increase in external KCl (the Na^+ level remaining constant), it is found that the Cl^- content of the cell increases, and that this increase is directly related to the outside level (*e.g.* external Cl^- 135 m.eq. per liter, increase in muscle 14 m.eq. per kg. external Cl^- 161 m.eq. per liter, increase in muscle 21 m.eq. per kg. Cl^-, equilibrated series, Table IV). However, the actual increase in the cell is greater than one would have expected from the increase in external concentration. This is shown by the fact that in all cases the ratio Cl^-_{out}/Cl^-_{in} is significantly lower in the treated muscles. This reduction in the Cl^- ratio was only seen in one out of six experiments when the Na^+ content of the Ringer was reduced in proportion to the added K^+, and consequently the Ringer Cl^- level remained the same.

The relationship between the Na^+ and Cl^- movements in high K^+ Ringer is extremely interesting. When the Na^+ level in the Ringer was not altered it was found (in the equilibrated series) that the Na^+ in the cell remained unchanged (the ratios are the same), whilst the Cl^- had more than proportionately increased, and consequently the ratio was less than in the control. In the unequilibrated series there was a significant loss of Na^+ (at 25 m.eq. per liter K^+ and higher levels), whilst the Cl^- had again entered the cell more than in proportion to the external increase. When the Na^+ level of the Ringer was reduced there was a disproportionate decrease in the Na^+ content of the treated muscles, but the Cl^- level (and ratio) did not alter in either the equilibrated or unequilibrated series (except for the one experiment mentioned above). Thus it is evident that the presence of a high external level of K^+ has altered the relationship between the movements of the sodium and chloride ions. In normal Ringer or when the NaCl level in Ringer was altered the movements of these ions were equivalent. From the results with high K^+ Ringer it would appear that the Na^+ and Cl^- ions can move independently. Thus the ratios Na^+ and Cl^- (out/in), which were previously found to be equal, have diverged under the influence of a high external K^+ level.

DISCUSSION

The work reported in this paper is an extension of that previously published (28) in which the intracellular changes of Na^+ and K^+ in the same muscle

were correlated with changes in the external ionic environment. These results may be summarised as follows:—

(*a*) Muscles soaked in K-free Ringer do not lose K^+ compared with companion muscles soaked in normal Ringer.

(*b*) When the external K^+ is raised stepwise to 100 m.eq. per liter a small amount of K^+ enters the cell.

(*c*) Whilst the amount of K^+ in the muscle *in vivo* is relatively constant there is great variation in the intracellular Na^+ level (0 to 70 m.eq. per kg.; *i.e.*, the ratio Na^+_{out}/Na^+_{in} varies from 1.85 to infinity). When the muscle is soaked in Ringer solution it loses or gains Na^+, until there is an approximately constant ratio of 3.

(*d*) If the external Na^+ level is raised or lowered (between 45 and 460 m.eq. per liter) Na^+ enters or leaves the cell to maintain this constant ratio.

(*e*) Rendering the Ringer solution hypertonic by the addition of KCl does not affect the volume of the cell. The addition or subtraction of NaCl to or from the Ringer solution (regardless of the K^+ concentration) results in a shrinkage or swelling of the cell. However, there is not a proportional relationship between the external NaCl level and the volume change.

In this paper we have repeated the previous work, but in addition we have analysed the same muscle for chloride (as well as Na^+ and K^+). We have confirmed the above results with respect to Na^+ and K^+. It remains to discuss the conclusions which may be drawn from the relationship between the movements of the three ions. This will be done under four headings.

1. Donnan Theory.—The accumulation of K^+ in the cell has been held in the past to be due to a Donnan distribution (2). Boyle and Conway (2) had found that an increase in external K^+ level resulted in an elevation in internal K^+ which agreed with that theoretically predicted. We are unable to confirm this (see Tables IV and V). Furthermore they stated (2) that in a K-free solution K^+ would leave the cell as long as it could be accompanied by Cl^-. We had shown (29) that under these conditions K^+ did not leave the cell, and we have now demonstrated that there is adequate Cl^- to accompany the K^+ into K-free Ringer (see Table IV). It is a fundamental requirement of the Donnan theory that the product $K^+_{in} \times Cl^-_{in} = K^+_{out} \times Cl^-_{out}$. Conway found that this relationship did not hold in normal Ringer, but only when there was an elevated K^+ level, or in the intact animal. The agreement with the Donnan ratio in muscles *in vivo* must be regarded as fortuitous, for those muscles with a low Cl^- (and Na^+) content will tend to give correct equality, but high Cl^- (and Na^+) muscles will not. This lack of correlation between K^+ and Cl^- *in vivo* is shown in Fig. 2. We were also unable to confirm this relationship in high K^+ Ringer, since there was an insufficient increase in intracellular K^+, with respect to the raised external level.

A further consequence of the Donnan theory is that there should be reciprocity between Na^+ and K^+ content. If the electrochemical gradient for K^+ is maintained by the active extrusion of Na ions, then high internal Na^+ levels should result in low K^+ levels, and conversely. It may be seen (Figs. 3 and 9) that such a correlation *in vivo* or in the isolated preparation does not exist.

We were also unable to reconcile the changes in the ionic content of muscle on soaking in normal Ringer with a Donnan mechanism. As was stated when discussing the absolute levels of intracellular ions, there should be a correlation between gain of Na^+ and loss of K^+, and a correlation between gain of Cl^- and loss of K^+. The lack of such a relationship is seen in Figs. 5 and 6.

The simple concept that the accumulation of K^+ was due to a Donnan mechanism with the Na^+ playing the role of an impermeant cation was proved untenable, because radioactive Na^+ was shown to cross the membrane freely. Since then the situation has become confused. Dean (5) introduced the concept of a Na^+ pump to account for the Na^+ permeability; following this the concept of a pump or carrier has been used to explain the movements of every cation and anion so far considered (6, 12, 20). It is difficult to ascertain at the present time to what extent the accumulation of K^+ is considered to be due to a Donnan mechanism bolstered by a Na^+ pump, with the passage of K^+ across the membrane facilitated by a carrier, and to what extent the accumulation is considered to be due to active transport alone. It would seem that the evidence cited in this paper must refute the Donnan concept.

2. Linked Carriers.—Hodgkin and Keynes (12, 13) have stated that the recovery movements of Na^+ and K^+ in squid axon after stimulation are coupled, though they do not seem to be rigidly linked. In our experiments with resting muscle we find little correlation between the movements of Na^+ and K^+.

With one exception, the movements of ions studied by us have been along concentration gradients, in the resting state. Thus our results are not strictly comparable with those obtained after the passage of an impulse in the squid axon. However, examples of non-reciprocal movement may be seen in Tables II and III and Fig. 6.

Keynes (16) found that placing a muscle in K^+-free Ringer reduced the Na^+ efflux. Hodgkin and Keynes (12) confirmed this finding in nerve. We have found that under these conditions the total Na^+ content of the muscle did not alter, and therefore if there was a decrease in efflux, there must also have been a decrease in influx. As the K^+ content of the cell after 4 hours soaking in K-free Ringer is the same as that of the companion immersed in Ringer, this suggests that K^+ efflux has remained the same as in normal Ringer, until a steady state is achieved, when efflux would appear to cease. (See also reference 29.)

When a muscle which has been soaked in Ringer is transferred to half Na^+ Ringer there is an extrusion of Na^+ against a concentration gradient (26). There is no concomitant movement of K^+. We have now shown that the Na^+

which leaves the cell is accompanied by Cl^-. Thus we have an example of the active transport of NaCl which is analogous to that performed by the frog skin (for bibliography see Ussing (33)). The analogy is not complete, because with the frog skin we are dealing with a multicellular membrane bathed on either side by a Ringer type solution. It is possible to postulate a net ionic transfer from one solution to the other taking place at a constant rate, and so fulfilling the conditions of the steady state. In this case the flux ratios will not be unity, but the assumption of a steady state justifies the calculations given by Ussing on page 9 of his paper (33). From a consideration of the flux ratios and the electrochemical gradient for the ion, he states that Na^+ is actively transported through the frog skin, and Cl^- follows passively. Unfortunately, one cannot transfer this process of reasoning to a system such as nerve or muscle where one is dealing with the flux across the membrane of a cell or group of cells, and the existence of a flux ratio which is not unity implies that the system is not in a steady state.

3. The Relationship between Na^+, K^+, and Cl^-.—As we have mentioned earlier in this discussion most workers have suggested that movements of K^+ and Cl^-, and K^+ and Na^+ are related. Our results show that there is not a first order correlation between these ions. On the other hand we have demonstrated a definite linkage between Na^+ and Cl^-.

In the intact animal there is a highly significant correlation between intracellular Na^+ and Cl^-. This is shown in Table I and Fig. 1. There is a significant correlation between Na^+ and Cl^- content in two sartorii taken from the same animal. It has also been shown that the ratio of Na^+ to Cl^- in muscle is the same as this ratio in plasma. This relationship is unaffected by alterations in the level of internal K^+.

The ion shifts found on soaking muscle in normal Ringer also reveal a highly significant correlation between Na^+ and Cl^- movements. The gains of Na^+ and of Cl^-, although closely related, are not equivalent. For each Cl ion entering the cell approximately 1.4 Na ions enter. As can be seen from Figs. 5 and 6 there is no correlation between Na^+ gain and K^+ loss, and Cl^- gain and K^+ loss.

There is also a highly significant correlation between Na^+ and Cl^- content of muscles soaked in normal Ringer. The ratio of Na^+ to Cl^- in muscle is, however, significantly different from the ratio of Na^+ to Cl^- in Ringer. The relationship is such that one Cl ion is associated with 1.7 Na ions. The results shown in Fig. 7 were divided chronologically into two series. In the first series the intracellular K^+ was abnormally low in several muscles (less than 55 m.eq. per kg.). A multiple correlation performed on this series showed that the Cl^- level was influenced by both the Na^+ and K^+ content. In the second series, in which these abnormal K^+ levels were not present, the intracellular Cl^- value depended only on the amount of intracellular Na^+.

When the external NaCl is raised or lowered the intracellular level of these ions follows the external change so that the ratio Na^+ or Cl^- (out/in) remains constant at approximately 2–3, and is the same in each individual muscle. There are no accompanying changes in internal K^+.

The only condition under which the equivalence of Na^+ and Cl^- ratios is not observed is that obtaining when the external K^+ is increased. Under these circumstances the Na^+ ratio tends to be increased, and the Cl^- ratio decreased; *i.e.*, there is less Na^+ than Cl^- in the cell.

4. Theoretical Considerations.—Contrary to currently held opinion there is a close correlation between Na^+ and Cl^- content, and no first order relationship between these ions and K^+. These findings have led us to consider a new concept of the distribution of ions within the cell. The nature of this concept is suggested by the results given in this paper, but must remain tentative pending the completion of confirmatory experiments.

In an earlier paper, Shaw and Simon (25) discussed the possibility that the accumulation of K^+ could be accounted for by an adsorption hypothesis. The suggestion that K^+ is adsorbed by the cell is not new (for literature see reference 25). It is no more unreasonable to assume that ionic differentiation takes place in the cell interior than that it happens within the mural structure.

The adsorption of K^+ cannot be considered to occur as the result of "classical ionic" forces; *i.e.*, it cannot be pictured as a point charge held in position under the action of electrostatic forces. If this were so then no discrimination between Na^+ and K^+ would be possible. The introduction of the concept of ionic radius raises further difficulties, for example the possibility of induced polarisation of the charge in the presence of an electric field. Indeed, it is logically inconsistent to conceive of a homogeneous sphere of charge, with an invariant shape, structure, and size, which can react as a whole with an electric field. It is clear that the radius of the ion is not invariable, but must be considered as an "interaction radius," resulting from the interaction of a particular ion with a particular environment. It is thus a quantity of secondary rather than primary significance. In this respect our views differ from those of Ling (19).

The binding of K^+ must thus be the result of a non-classical process such as electron exchange, which gives rise to a covalent type of directed binding force. This will be strong, and of very short range compared with the range of electrostatic forces. The current physical picture of the ion, with a distributed charge density whose details depend on the electrodynamics of the situation, and whose charge density will differ between equivalent ions (*i.e.*, Na^+ and K^+), gives a possible mechanism for ionic discrimination. Thus it is postulated that K^+ is adsorbed onto the ultrastructure of the cell. The K^+ sites will be highly specific, and the normal metabolic function of the cell is necessary to maintain the sites, which may be cyclically broken down and regenerated. The K^+ sites are situated in a charged, semirigid, ordered lattice structure which

occupies about two-thirds of the cellular volume. (For a general discussion of polyelectrolyte gels see Katchalsky (15).) Na^+ and other ions not associated with the structure of the lattice are excluded from the region it occupies. The other excluded ionic constituents diffuse into the remaining smaller fraction of the cell which is in physicochemical equilibrium with the Ringer. The isolation of these ions in the smaller space results in an apparent concentration gradient across the membrane when their concentration is expressed in terms of total cell volume.

The evidence for the existence and extent of this space is as follows: (*a*) Na^+ and Cl^- movements are equivalent in the majority of our experiments. (*b*) The ratio of Na^+ and Cl^- (out/in) is constant, and on the average has a value of 3. Thus the extent of this space is one third of the total cell volume. (*c*) LiCl shows the same relationships (unpublished observations). (*d*) There will also be a small amount (about 1 m.eq. per kg.) of "free" K^+ in this space in equilibrium with the Ringer. When a muscle is placed in high K Ringer there is a small gain in K^+, which is directly related to the external level. Since this increase represents diffusion into the free intracellular phase (F.I.P.) then the *increase* in K^+, compared with the controls, should be in the same ratio (K^+_{out}/K^+_{in}) as is found with the other ions. This calculation will be complicated by the fact that the increase in external K^+ is known to reduce the loss of K^+ found on soaking in normal Ringer (Table IV). Thus the increase in intracellular K^+ in the unequilibrated series will represent a reduction in this loss, plus diffusion into the free intracellular phase. If one considers the equilibrated series, in which this loss will take place during the equilibration period (Tables IV and V), then it is apparent that the increase in K^+ agrees reasonably well with that calculated. For example in Table IV (equilibrated series) the gains of K^+ at 25, 50, and 87 m.eq. per liter external level are 10 m.eq. per kg. K^+ found $\left(8 \text{ m.eq. per kg. calculated from } K^+ \text{ out} \div \frac{Cl^-_{out}}{Cl^-_{in}}\right)$, 20 m.eq. per kg. found (17 m.eq. per kg. calculated), and 62 m.eq. per kg. found (40 m.eq. per kg. calculated). In Table V the values at 25 and 50 m.eq. per liter external K^+ are 9.0 m.eq. per kg. found (6 m.eq. per kg. calculated) and 16 m.eq. per kg. found (16 m.eq. per kg. calculated.) When the increase in intracellular K^+ is compared with controls *in vivo* (Table VI) there is an actual addition of 15 m.eq. per kg. compared with a calculated increase of 17 m.eq. per kg. The undue increase found at 87 m.eq. per liter may reflect a reversal of the loss found on soaking in normal Ringer; *i.e.*, there may have been a reconstitution of adsorption sites. This would result from a decrease in the energy required to maintain a site, following a decrease in the K^+ gradient. With this one exception at 87 m.eq. per liter the differences between the found and calculated values are not statistically significant.

There are two apparent inconsistencies with the simple theory set out above.

The ratio of Na^+ and Cl^- (out/in) in the living animal is usually much greater than three, and is subject to great variation. It is now necessary to explain why the NaCl enters the muscle on soaking to give an approximate ratio of three. It is possible that the semirigid lattice structure wherein the K^+ sites are situated extends to a larger extent throughout the cell *in vivo*, and so excludes a greater amount of Na^+ and Cl^-. When the cell is removed from the body there will be a decrease in available energy to support this semirigid structure, which becomes gradually disordered. This loss of order within the cell permits the entry of a certain amount of NaCl. After about an hour a new balance is set with the available energy, and a steady state is achieved. This state is maintained for at least 8 hours, but as the cell dies a further disintegration of structure takes place, and more NaCl enters until finally the ratio approaches unity on a fiber water basis (Shaw and Simon (25)).

The ratio of Na^+ to Cl^- in the intact animal is the same as the ratio of Na^+ to Cl^- in plasma. However, when the muscle is soaked in normal Ringer there is an excess of Na^+ over Cl^- in the free intracellular phase (see Fig. 7), the additional Na^+ may preserve electroneutrality if there is an accumulation of phosphate or bicarbonate. Contrary to the above finding there is a higher ratio Na^+_{out}/Na^-_{in} than Cl^-_{out}/Cl^-_{in} when muscle is soaked in high K^+ Ringer. Both these points will be discussed in a later paper. In other words our results illustrate that the movements of Na^+ and Cl^- are passive and depend on the external concentration. To account for this we have suggested that the cell consists of two parts, first, there is an ordered semirigid gel, which specifically adsorbs K^+, and excludes other ions. Second, there is a small and relatively disordered free space within which other ions exist in equilibrium with the extracellular phase. It is quite possible that indiffusible ions exist within this free space and therefore will influence the distribution of diffusible ions according to Donnan principles. When the cell is removed from the body and the energy available to maintain the ordered structure decreases, the disordered phase will grow at the expense of the ordered phase and ions, particularly Na^+ and Cl^-, will enter from without. This free space may not necessarily be coaxial with the ordered phase, but may in parts exist within it.

This theory replaces a mural mechanism for the separation of ions with one dependent on the structural integrity of the whole cell. Cowie and Roberts (4), using an approach similar to our own, have come to the conclusion that the cell membrane plays a very minor role in the maintenance of the ionic pattern in *E. coli*. They state that it must be regarded as a morphological boundary, permitting the free access of all molecules of dimension smaller than that of proteins. The consequences of our theory for the interpretation of the bioelectric potentials will not be dealt with fully in this paper. Briefly, if the Na^+ and Cl^- are in equilibrium across the membrane it would be difficult to see how they could influence the resting or action potential as a first order. This

would be in accordance with the results presented earlier (28). The assumption of an equilibrium between the Na^+ and Cl^- in the free intracellular phase and that in the external solution obviates the necessity to postulate a sodium pump in resting muscle.

Other authors have suggested that there is a separate space or compartment within the cell, in which the ionic content differs from that of the remainder of the cell. Shanes and Berman (23) have postulated such an "X-phase" in the giant axon. The existence of two intracellular compartments in red blood cells has been suggested by Solomon and Gold (30). They leave the nature of the compartment unspecified, but suggest it contains K^+ at a much lower concentration than the major portion of the cell and that the concentration of K^+ in the smaller compartment is about equivalent to that in blood. Carey and Conway (3) from their results with radioactive Na^+ and K^+ suggest that most of the Na^+ of muscle is in a region with a high permeability to both Na^+ and K^+, and with concentrations of Na^+ higher, and K^+ lower than in other parts of the fiber. These authors have calculated that the amount of Na^+ in the special region is 8 m.eq. per kg., which would agree with our calculation of the Na^+ in the F.I.P., in many muscles in the intact animal. As we have said, the F.I.P. increases when the muscle is soaked in ordinary Ringer. However, we think there would be much less than 1.75 m.eq. per kg. K^+ in this space; *i.e.*, $2.5 \times \frac{8}{104} = 0.19$ m.eq. per kg. (see page 248 of original reference (3)). Carey and Conway noticed that the rate of exit of Na^+ from sartorii when immersed in isotonic glucose containing 100 mM KCl or 5 mM K_2SO_4 per liter was the same. They then say that this supports the view that the Na^+ exchanges with the K^+. Our opinion is that it is not an exchange, but a simple diffusion down a concentration gradient, and that the movements of the two ions are in no way linked. If there were a connection, the rates of Na^+ exit would be unlikely to be the same in the two different K^+ solutions.

The inhomogeneity of cells has also been stressed by Bartley *et al.* (1), who state:

"Apart from the outer boundaries (of the cell) there are many intracellular boundaries which behave like semipermeable membranes. This applies in particular to the boundaries of mitochondria. Mitochondria are compartments within a compartment, and it is very probable that mitochondria are further subdivided into smaller compartments, separated by permeability barriers. The nucleus is another subunit within the cell, separated from the rest of the cell by membrane-like barriers. These intracellular permeability barriers may be no less important than those of the outer cell wall." In muscle the highly organized structure of the contractile mechanism must also be considered in this regard. It is tempting to identify the ordered phase containing adsorbed K^+ with the "organoids" of the cell (mitochondria, etc.) and the free intra-

cellular phase with the interstices, but this would no doubt be an oversimplification. For example Itoh and Schwartz (14) have shown that nuclei contain appreciable amounts of Na^+, and it is not at present known whether this ion is in a diffusion equilibrium with the exterior (and therefore logically part of the F.I.P.) or adsorbed onto the structure of the nucleus.

SUMMARY

The $Na,^+$ Cl^-, and K^+ content of toad plasma and the sartorius muscle has been determined. Although the Na^+ and Cl^- level of the muscles in the living animal varied greatly (0 to 38.0 m.eq. per kg., and 0 to 31.8 m.eq. per kg. respectively) the K^+ level was subject to a smaller variation (76.5 to 136 m.eq. per kg.). There was a direct relationship between Na^+ and Cl^-, which was independent of the K^+ level.

There is a closely related gain of Na^+ and Cl^- when muscle is soaked in normal Ringer. These gains are not related to the K^+ loss, frequently found on soaking.

The relationship between the three ions was studied in a large series of 124 muscles in normal Ringer. As found *in vivo*, there was a correlation between Na^+ and $Cl.^-$ This correlation was independent of K^+ content, except when this was abnormally low.

Alteration of the external NaCl level produced concomitant changes in the internal levels of these ions.

Alteration of the external KCl level produced an increase in internal Cl^- similar to that found with high NaCl solutions, but the amount of K^+ entering the cell was approximately one-third of the external increase.

Removal of K^+ from the external solution did not result in a loss of K^+ from the cell, although there was an adequate amount of Cl^- present to accompany it.

The results cannot be reconciled with either a Donnan concept for the accumulation of K^+, or a linked carrier system.

A theory is proposed to account for the ionic differentiation within the cell. The K^+ is assumed to be adsorbed onto an ordered intracellular phase. The normal metabolic functioning of the cell is necessary to maintain the specificity of the adsorption sites. There is another intracellular phase, which lacks the structural specificity for K^+, and which contains Na^+, Cl^-, and K^+ in equilibrium with the external solution. The dimensions of the free intracellular phase will vary from cell to cell, but it will be smaller in the intact animal, and will increase on soaking in normal Ringer, until it is approximately one-third of the total cellular volume. The increase in this phase may be ascribed to a decrease in the energy available to maintain the ordered phase.

We are very grateful to Professor M. Belz and Mr. Rupert Leslie for carrying out the statistical analyses. We wish to acknowledge the help of Miss Alma Werner, M.Sc., with certain theoretical considerations.

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LACTIC AND α-GLYCEROPHOSPHATE DEHYDROGENASES IN INSECTS

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(Received for publication, December 27, 1956)

INTRODUCTION

The muscles of insects are among the most efficient known in the animal kingdom. They are able not only to contract at a very high rate (up to 1000 contractions per second), but also to work continuously for long periods. Studies on their biochemistry would seem to promise results of general interest. Up to the present time, however, only a few workers have been attracted to the study of insect muscle and our knowledge is accordingly very meagre. The results of the first detailed experiments in this field have not been particularly encouraging and they have failed to reveal significant differences in the biochemistry of insect and vertebrate muscle. As is true of mammalian muscle, the action of the flight muscles of bees and flies depends on a sufficient supply of carbohydrates, which has been demonstrated in elegant experiments by Beutler (1936, 1937) and Wigglesworth (1949). In recent years, however, evidence has been found that the metabolism of other insects—probably of the majority—differs distinctly from that of the vertebrates. Locusts, for example, are able to utilize fats exclusively as energy source for their muscular work, especially during prolonged flights (Krogh and Weis-Fogh 1951, Weis-Fogh 1952). Lepidoptera seem to be even more specialized in this direction. Probably they do not utilize carbohydrates directly to a significant extent, but transform them into fats before utilization (Zebe 1953, 1954). Between these two types, namely, insects which use carbohydrates and those which use fats, others take an intermediate position, being able to utilize both fats and carbohydrates.

Because of such variation in their metabolism insect muscles studied biochemically from the comparative point of view seemed to promise rewarding results. It was considered to be of particular interest to find out whether some of the great anatomical differences between insects and vertebrates might correlate with different conditions for the action of the muscles in the two groups. These conditions might possibly result in basic differences in the biochemistry of these animals. Very little attention has been given to this problem in the past. The basis for the studies which will be reported here, therefore, involves

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the following questions: (1) Does the group of insects in general possess a glycolytic system as it is known in the vertebrates? (2) Is there an indication that during periods of hard work their muscles also suffer from a shortage of oxygen? (3) Do biochemical differences or modifications exist which are specific to the two types of metabolism in insects?

Material and Methods

The insects for the experiments were raised in the laboratory or caught outdoors. They were dissected in the cold. If their size was too small to allow proper separation of the different tissues, thorax or abdomen was used as a whole after removal of the limbs. Experiments on whole animals were done in only a few cases (larvae).

The blood substitute "periston" (Bayerwerke) which contains about 4 per cent polyvinyl pyrrolidone was used for the extraction of the tissues or the suspension of the homogenates; it has been proved to be an excellent medium for making tissue preparations. Phosphate was added to make a 0.05 M solution of pH 7.3. The procedure for the extraction was as follows: The tissue was homogenized in ten volumes of the periston-phosphate solution in a sharply pointed ground glass homogenizer, centrifuged (5 to 10 minutes at 17,000 × *g*), and the clear supernatant fluid separated. The residue was subjected to this procedure four more times including homogenization of the tissue each time with new fluid.

The activities of the DPN-linked enzymes were measured by means of the optical test. This assay is based on the rate of oxidation of reduced DPN under precisely standard conditions (Beisenherz *et al.*, 1953) indicated by decreasing absorption of ultraviolet light at a wave length of 340 mμ. The enzymic activity is expressed in units as they were defined by Bücher and coworkers (Beisenherz *et al.*, 1953). One unit corresponds to a turnover of 1.76×10^{-8} M reduced DPN per minute per ml. A Beckman model DU spectrophotometer was used. The test medium was 0.1 M triethanolamine hydrochloride buffer of pH 7.56. The test volume was 1 ml. in a cuvette of 10 mm. diameter. The concentration of the enzymes was varied according to their activity between 0.002 and 0.070 mg. protein per cuvette.

The Warburg experiments were done in air at 38°C. using conventional apparatus and flasks. A modification of the biuret method (Beisenherz *et al.*, 1953) was used for the protein determinations. Lactate was determined by means of the colorimetric method of Barker and Summerson (1941).

RESULTS

The efficiency of the extraction of the enzymes from insect muscle was studied before assaying the extracts quantitatively for their enzymic activities. Woodroach thoracic muscle was used for this purpose. It was extracted five times with the same volume of fluid and the extracts were tested separately for their glycerophosphate dehydrogenase activity. The protein contents were determined simultaneously. The data in Table I show that the enzymic activity decreased rapidly from the first extract to the fifth which did not contain significant activity. The protein content decreased at a slower rate than the

enzymic activity. These results demonstrate the high water solubility of GDH I(DPN-linked) from insect muscle and show that it is removed readily from the tissue by five times repeated homogenization and extraction.

The oxidation of reduced DPN by extracts from insect tissues was studied first in the absence of any substrate. In all cases it was found to be extremely low. An extract of *Phormia* flight muscle (0.016 mg. protein/cuvette), for instance, which required 20 seconds to decrease the extinction (log I_o/I) by 0.100 (Δ 100) in the presence of dihydroxyacetone phosphate (standard conditions for GDH I assay) required 1800 seconds without substrate in the control experiment.

Flight Muscle.—The results of preliminary experiments showed surprisingly that the flight muscles of honey-bees and locusts, insects chosen as examples of the two different metabolic types, contain very little lactic dehydrogenase

TABLE I

GDH I Activity and Protein Content in the Different Extracts from Wood-roach Thoracic Muscle (Leucophaea maderae)

Extract No.	Enzymic activity		Protein content		Units/mg. protein
	units/ml.	*per cent*	*mg./ml.*	*per cent*	
1	1540	89.4	1.580	61.3	975
2	154	8.9	0.416	16.2	370
3	23	1.3	0.272	10.6	85
4	5	0.3	0.170	6.6	29
5	2	0.1	0.136	5.3	15
	1724	100.0	2.574	100.0	

(LDH). The similar results obtained with the two insects suggested that the absence of LDH activity might be a feature characteristic of insects in general and perhaps be related somehow to the different way that oxygen is supplied to the tissues of these animals. In order to obtain further evidence in support of this hypothesis the studies were extended to a larger number of insects belonging to as many different families as possible. The results of these experiments show that the activity of LDH is extremely low in the flight muscles of all insects studied. A significant level was found only in a few cases. The examples of the rat leg muscle and the abdominal muscle of the crayfish (bottom of Table II) demonstrate the striking difference between the respective muscles of these animals and the flight muscles of insects.

The results of the same experiments revealed another peculiar feature of insect flight muscle, namely, its extraordinarily high content of α-glycerophosphate dehydrogenase (GDH I, DPN-linked). Although the data show variations in the different species (for which the different degree of physical activity

of the specific animals might be responsible), the activity of GDH I always exceeded that of vertebrate muscle many fold. It appears that the proportions of LDH and GDH I activities in flight muscles of insects are reversed from those found in mammalian muscles.

Leg Muscle.—The LDH activity of extracts from leg muscle was usually as low as in thoracic muscle. In two cases, however, a very active LDH was found (Table III). It is of interest that in these two insects the legs perform especially hard work: an efficient rowing stroke in the water-bug *Belostoma* and the extremely strong contraction necessary for the jumping of the grasshopper

TABLE II

Enzymatic Activities in Extracts from Thoracic Muscles of Some Insects

	GDH I* (DPN)	GDH II‡ (cytochrome)	LDH*
Phormia (Diptera)	613	82	4
Apis	800	110	3
Bombus (Hymenoptera)	724		>3
Leucophaea (Blattidae)	647	55	4
Melanoplus (Orthoptera)	313	56	40
Lestes (Odonata)	164		10
Libellula (Odonata)	278		11
Telea (Lepidoptera)	200	23	7
Agrotis (Lepidoptera)	652		7
Gyrinus (Coleoptera)	574		19
Pelidnota (Coleoptera)	477		32
Tetraopes (Coleoptera)	498		9
Cambarus (abdominal muscle)	6		247
Rat (leg muscle)	29		440

* Units per milligram protein.

‡ Cubic millimeters oxygen per milligram protein per hour.

(*Melanoplus*). Although the activity of GDH I in leg muscle is generally somewhat lower than in thoracic muscle, it is still high in comparison to animals from other groups. It is interesting that in the two cases of exceptionally high LDH activity the GDH activity seems to be decreased to a certain degree. A comparison of the data from the grasshopper leg muscle (Table III) with those from its flight muscle (Table II) demonstrates clearly how striking the differences can be between the specific muscles of the same animal.

Other Tissues and Larvae.—Some determinations were also made of the GDH I and LDH activities of extracts from other tissues of the insects. Both enzymes were found to be practically absent from the gonads. Significant LDH was not present in the fat bodies of the species studied with the exception of the blowfly *Phormia* which showed slight LDH activity. However, GDH was

rather active in the fat bodies of the insects although there was considerable difference among several of the species (Table IV).

Somewhat less distinct are the results obtained in experiments on insect larvae in which the whole animal was homogenized. In the extracts of two

TABLE III

Enzymatic Activities in Extracts from Leg Muscles of Insects

	GDH I* (DPN)	GDH II‡ (cytochrome)	LDH
Bombus (Hymenoptera)	204		>3
Tetraopes (Coleoptera)	29		3
Pelidnota (Coleoptera)	44		3
Belostoma (Hemiptera)	16		139
Melanoplus (Orthoptera)	193	36	187

* Units per milligram protein.

‡ Cubic millimeters oxygen per milligram protein per hour.

TABLE IV

Enzymatic Activities in Extracts from Other Tissues of the Insect Body

	GDH I* (DPN)	GDH II‡ (cytochrome)	LDH
Fat body			
Phormia§	39	17	14
Apis‖	29	0	0
Leucophaea	45	0	3
Melanoplus	88	0	3
Telea	45	0	3
Testes			
Leucophaea	3		1
Ovaries			
Telea	3		1

* Units per milligram protein.

‡ Cubic millimeters oxygen per milligram protein per hour.

§ Whole abdomen.

‖ Whole abdomen after removal of gut.

species the GDH activity exceeded by far the LDH activity. However, the example of *Prodenia* shows that a great part of the activity is located in the fat body. In the blowfly maggot and in the chironomid larvae the proportions are reversed (Table V).

The data from the first assays of the blowfly tissues were not quite satisfactory because they showed wide variations. It was suspected that different ages

might be responsible for these results. Experiments were done, therefore, with flies of known age which ranged from individuals just emerged to those 3 weeks old. The values in Table VI show that great differences do exist. Within the first 10 days of adult life there is a fourfold increase in GDH I activity in the flight muscle which remains constant afterwards. In the fat body (abdomen)

TABLE V

Enzymatic Activities in Extracts from Insect Larvae

	GDH I (DPN)	LDH
Chironomus	3	15
Phormia	11	28
Tenebrio	9	0
Prodenia (whole)	48	13
Muscle	28	14
Fat body	68	13

Units per milligram protein.

TABLE VI

Enzymatic Activities in Extracts from Blowflies of Different Age

Age	Thoracic muscle			Abdomen		
	GDH I* (DPN)	GDH II‡ (cytochrome)	LDH*	GDH I* (DPN)	GDH II‡ (cytochrome)	LDH*
days						
1	154		14	7		1
3	325	24	4	34	6	4
6	475	72	5	43	15	12
10	637	85	10	26	39	11
14	612	82	4	39	17	14
21	630	77	5	43	17	11

* Units per milligram protein.

‡ Cubic millimeters oxygen uptake per milligram protein per hour.

GDH I reaches its peak somewhat earlier. This applies also to the LDH of this tissue.

Particulate Glycerophosphate Dehydrogenase (GDH II).—The residues of the tissues which had been homogenized and extracted five times were examined for possible remaining enzymic activities. It was found in Warburg experiments that some of these preparations were still able to oxidize α-glycerophosphate. By addition of cytochrome *c* the oxidation was increased by five or six times. The addition of DPN did not result in any increase in oxygen consumption (Fig. 1). Furthermore, the pH optimum of the reduction of dihydroxyacetone

phosphate under the conditions of the optical test and that of the glycerophosphate oxidation by the residue of thoracic muscle differed to some degree. The first was determined to be sharply pH 6.4; the second was found to lie less distinctly between pH 6.7 and 6.8. The fifth extract as shown above in Table I did not contain significant GDH I activity. It was concluded on the basis of these results that a second glycerophosphate dehydrogenase (GDH II) is present which is not extracted from the tissue and which is not DPN-specific, but which is linked directly to the cytochrome system as is the case with succinic dehydrogenase. A particulate GDH of this kind has been previously reported by Green (1936) to occur in vertebrate muscle (see also Ta-Cheng Tung *et al.*,

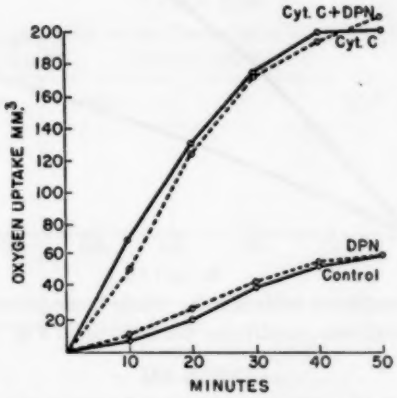

FIG. 1. α-Glycerophosphate oxidation by muscle residue. Tissue, thoracic muscle of *Leucophaea*. Each Warburg flask contained 0.5 ml. of a 10 per cent suspension of the residue; 4.3×10^{-6} M 1-α-glycerophosphate. Final concentrations of cofactors, 6×10^{-6} M cytochrome *c*; 8×10^{-4} M DPN.

1952). Its presence in insects was demonstrated in both flight and leg muscle, while its distribution in other tissues is still uncertain (Tables II to IV). Significant activity of GDH II, however, was found in the fat body of the blowfly.

Since the oxygen uptake of the residues was great, it was of interest to determine to what extent the particulate GDH is involved in the oxidation of glycerophosphate by the whole homogenate. In this connection the oxygen consumption of the residue and of the whole homogenate of blowfly flight muscle were compared under exactly the same conditions. It was found that the whole homogenate took up almost three times as much oxygen as the residue (Fig. 2). This might be interpreted as indicating that the GDH II accounted for one-third of the glycerophosphate oxidation. It appears very questionable, however, whether these two enzymes were both working under optimum conditions. It is conceivable that the optimum conditions for one of these enzymes might

possibly affect the activity of the other. In addition, it is not known to what extent further oxidation of the products added to the oxygen consumption of

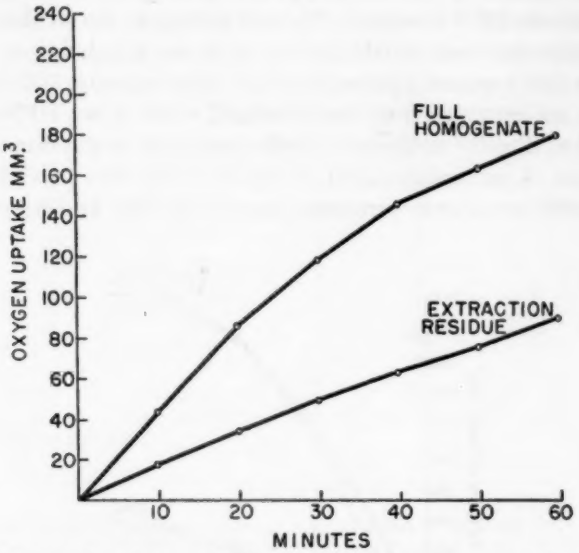

FIG. 2. α-Glycerophosphate oxidation by whole homogenate and residue. Tissue, thoracic muscle of *Leucophaea*; conditions the same as in Fig. 1.

TABLE VII

Production of Lactate by Homogenates of Different Muscles under Anaerobic Conditions

	Lactate		
	Control	After 1 hr.	Increase
	mg. per gm. tissue	*mg. per gm. tissue*	*mg. per gm. tissue*
Leucophaea maderae (thoracic muscle)	1.12	3.05	1.93
Telea polyphemus (thoracic muscle)	2.96	5.20	2.24
Melanoplus differentialis (femur muscle)	3.50	10.00	6.50

Each Thunberg tube contained: 2.0 ml. homogenate (50 to 100 mg./ml.) in 0.1 M phosphate buffer pH 7.3 + 0.1 KF; 0.0050 M magnesium chloride, 0.0038 M fructose diphosphate, 0.0030 M pyruvate, 0.0050 M ATP, 0.0004 M DPN, 0.0050 M glucose (all final concentrations), 0.1 M phosphate buffer was added to make a total volume of 6 ml.

the whole homogenate. In consideration of these conditions the results must be interpreted with caution.

Lactate Production.—Although the results obtained with the optical tests seemed reliable, it was thought desirable to check them by means of another

method. In this connection the formation of lactate from a suitable substrate by muscle homogenate was studied under anaerobic conditions. If the activity of LDH in flight muscle was almost lacking as indicated by the results given above, only a very small amount of lactate would be expected to be formed. On the other hand, grasshopper leg muscle with its much higher LDH activity should metabolize more substrate to lactic acid. Indeed these postulates were proved true, although the differences between the two kinds of muscle were not quite as great as was expected from the LDH activities. Thus in the experiments with grasshopper leg muscle three times as much lactate accumulated as in those with flight muscle (Table VII).

TABLE VIII

Influence of Different Substances on the Production of Lactate by Homogenate of Blowfly Thoracic Muscle

FDP	Glucose	ATP	GP	LDH	Lactate
					mg. per gm. tissue
+	+	+	−	+	12.7
+	+	+	+	−	2.6
−	−	−	+	+	10.6
−	−	+	−	+	2.0
−	+	+	−	+	12.6

Concentrations the same as in Table VII.

FDP = fructose diphosphate.
ATP = adenosinetriphosphate.
GP = α-glycerophosphate.
LDH = lactic dehydrogenase.

Experiments were also done in which traces of a pure LDH preparation from mammalian muscle were added to the muscle homogenate and substrate. It resulted in a great increase in lactate production (Table VIII). These findings indicate that the LDH activity actually is the limiting factor of the system while the other enzymes involved in the breakdown of carbohydrates by the Embden-Meyerhof pathway are working satisfactorily.

DISCUSSION

The reported experiments have established the fact that LDH activity is almost absent from insect flight muscle. The conclusion drawn from this finding is that these muscles do not possess the typical glycolytic system of vertebrate skeletal muscle. The mechanism of glycolysis enables the muscle to produce energy by means of a partial oxidation of glycogen or glucose even under anaerobic conditions. It is unable to function if the absence of LDH inhibits the formation of lactate from pyruvate. The presence and probably rather high

activity of the other glycolytic enzymes do not mean a contradiction of this statement for they are necessary for the breakdown of carbohydrate on the ordinary pathway *via* pyruvate to complete oxidation in the citric acid cycle.

The exceptions, namely, high LDH activity in some leg muscles which indicates the presence of an efficient glycolytic system, may be considered as adaptions to conditions of insufficient oxygen supply from which flight muscles apparently do not suffer. Thus, in *Belostoma* a shortage of oxygen is highly probable, since this insect can take only a very limited amount of air while diving under the water surface during which time the leg muscles contract vigorously. The violence of contraction required in the leg muscle of the grasshopper must be extremely high momentarily, so that the supply of oxygen may not be sufficient to meet the demand. In addition, the route for transporting oxygen is extraordinarily long because the spiracle of the trachea which supplies the leg muscle is located in the thorax at some distance from the muscle itself. The hypothesis that glycolysis (high activity of LDH and production of lactate) in insects occurs only under conditions of low oxygen supply is also supported by the results of the experiments done on larvae. Thus the blowfly maggots and the chironomid larvae which contain a very active LDH live ordinarily under conditions of frequent oxygen shortage, namely, in decaying meat and in the mud on the bottom of lakes and streams, respectively.

Previous studies on the metabolism of insect muscle have not provided sufficient evidence to show the presence of the glycolytic mechanism in this tissue. The first workers in this field (Davis and Slater, 1928, and Blanchard and Dinulescu, 1932), who found the accumulation of some lactate in the bodies of roaches and *Gastrophilus* larvae, pointed out that the amount of this substance was much smaller than was to be expected from the decrease in glycogen and glucose content or the oxygen uptake of these insects. In this connection the accumulation of lactate in insect muscles or other tissues has not been reported after periods of prolonged exercise, long flight, for example.

The incubation of cockroach or locust muscle preparations with a suitable substrate revealed that only very small amounts of lactate were formed in absence of oxygen while in the case of the locust thorax muscle pyruvate seemed to be the main reaction product (Barron and Tahmisian, 1948, and Humphrey and Siggins, 1949).

The fact that Chefurka (1954) demonstrated the presence of LDH in houseflies does not contradict the results reported here because both sets of data show approximately the same low level of LDH activity.

Having found out that insect muscle does not possess the glycolytic system of the vertebrates, one asks what the meaning and interpretation of this result may be. In this connection it should be pointed out that very low LDH and high GDH activity were always found in the flight muscles whereas in some special cases of leg muscle the related activities were exactly reversed. This suggests that the combination of extremely low activity of one enzyme and

the abundance of the other may not be merely incidental, but may have significance from the physiological standpoint. It seems that in the different muscles, at least to a certain degree, one enzyme might take the place of the other. In the metabolic scheme of the vertebrates so far known the oxidation of glycerophosphate and the reduction of dihydroxyacetone phosphate are apparently rather unimportant side reactions. The high activity of GDH I and, in addition, the presence of a very active particulate GDH suggest that in insects these reactions must have physiological significance.

The absence of glycolysis from insect muscle could mean that there is little, if any need for an anaerobic mechanism because the tracheal system is so much superior to the respiratory system of the vertebrates in supplying oxygen that there is never a shortage. Immediate and complete oxidation of the substrates is accomplished, therefore, without accumulating them in a half-oxidized form; *i.e.*, as lactate or another intermediate substance. Such a hypothesis would account for a manifold energy output and offer a plausible explanation for the tremendous supply of energy required by the thoracic muscles during flight.

The results reported clearly show that there is remarkable variation in the metabolism even in a tissue of such uniform structure and function as skeletal muscle from animals which belong to different groups of the animal kingdom.

SUMMARY

Different tissues, especially muscles, from insects belonging to various groups were extracted and studied for their lactic dehydrogenase (LDH) and α-glycerophosphate dehydrogenase (GDH I) activities from the comparative point of view.

In all cases assays of flight muscle extracts showed extremely low values of LDH activity whereas the GDH activities were surprisingly high. The activities in leg muscles were generally lower. In some insects, however, a very active LDH was found; in these cases the GDH activity seemed to be decreased.

GDH I was rather active in the fat bodies.

The presence of particulate glycerophosphate dehydrogenase (GDH II) was also demonstrated in insect muscles.

These results were interpreted as indicating a system by which there is accomplished immediate and direct breakdown of metabolites to supply large amounts of energy during flight and other activities without the accumulation of intermediate substances.

Preliminary experiments for this paper, which will be published in connection with studies on other problems, were performed in the Department of Physiological Chemistry at the University of Marburg, Germany. They were made possible by the Deutsche Forschungsgemeinschaft.

The authors are greatly indebted to Professor Dr. Theodor Bücher, Marburg, for many valuable suggestions and his help.

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STAPHYLOCOCCAL VIROLYSIN, A PHAGE-INDUCED LYSIN

Its Differentiation from the Autolysin of Normal Cells*

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(Received for publication, December 31, 1956)

SUMMARY

Virolysin is a lysin which appears in *Staphylococcus aureus* K_1 cells infected with phage P_{14}; together with phage, virolysin is released from phage-infected cells at the time of lysis. Autolysin is a lysin formed by uninfected cells of the K_1 strain; autolysin is released from uninfected cells by autolysis.

They show the following similarities: Both agents act within the genus *Micrococcus*. They lyse cells only after the cell has been subjected to a damaging or "sensitizing" treatment, such as heat, bacteriophage, acetone, or ultraviolet irradiation. The course of lysis of heated cells by both lysins has been found to proceed in a similar manner. A constant percentage of cells is lysed, independent of the concentration of lysin; the residual cells remain resistant to either lysin. Lysis proceeds logarithmically with time, and the velocity constants K are proportional to the lysin concentration. K increases with increasing temperature.

Both lysins are unaffected by antiserum to the phage. They are inhibited alike by a number of chemicals, including known enzyme inhibitors. Both agents are destroyed by proteolytic enzymes and are precipitated by 40 per cent saturation with $(NH_4)_2SO_4$. Both lysins are very thermolabile.

The two lysins differ with respect to their pH optimum, antigenic relationship and specificity for *Micrococcus lysodeikticus*.

These results suggest that (1) both lysins have many properties associated with enzymes, (2) the lysis of heated cells, which they produce, has some of the characteristics of a chemical reaction, (3) the lysin from the phage-infected cell is clearly different from the lysin of the uninfected cell.

INTRODUCTION

A lysin has been detected in the supernatant layer of ultracentrifuged phage lysates of *S. aureus* K_7 and 145 (Ralston, Baer, Lieberman, and Krueger, 1955).

*This investigation was supported in part by grants from ONR and the Board of Research, University of California. Thanks are due to Dr. John H. Northrop for his generous advice.

The name virolysin has been proposed to designate those lysins which appear as a result of virus infection and are not detectable in uninfected host cells.

A number of authors have proposed that the autolytic systems of the host are involved in the lyses that result from various phage-host interactions (Bronfenbrenner and Muckenfuss, 1927; Wollman and Wollman, 1933; Huppert and Panijel, 1954; Puck, 1953). This paper is concerned chiefly with the relationship between the virolysin of phage lysates and the autolysin of normal host cells. The problems of the intracellular appearance of virolysin, its function in lysis-from-without and in the lysis of infected cells at the end of the latent period are described elsewhere (Ralston *et al.*, data to be published, 1957 *a*, *b*).

Materials and Methods

Host-Virus Systems.—These experiments have been performed with phages P_{14} and P_1 and hosts *S. aureus* K_1 and 145. The systems and the method of phage assay have been described (Jones and Krueger, 1951; Ralston and Krueger, 1952, 1954).

Media.—(1) Tryptose phosphate broth, Difco, 30 gm. per liter, (2) double strength tryptose phosphate broth, 60 gm. per liter, (3) tryptose phosphate agar, 30 gm. tryptose phosphate and 25 gm. agar per liter, (4) mineral salts broth, M/5000 Mn^{++}, Mg^{++}, and Ca^{++}, M/15 KH_2/K_2HPO_4, 0.14 M NaCl, and 3 gm. tryptose phosphate per liter.

Preparation of Heat-Killed Cells.—Cells were grown on tryptose phosphate agar at 37°C. Each plate was inoculated with 1×10^9 bacteria per ml. from a 16 to 18 hour slant of *S. aureus* K_1 (unless otherwise stated). Logarithmic phase cells were collected after 3 to 4 hours; resting phase cells after 16 to 24 hours. They were both harvested in 0.85 per cent saline, usually washed once, resuspended in saline to *ca.* 5×10^{10} cells per ml., and were killed by heating at 56–100°C. for over 30 minutes. Suspensions were then stored at 4°C.

Lysis Measurements.—Lytic activity was measured by turbidimetric determination of cellular lysis, using a Klett-Summerson photoelectric colorimeter. For example, 5 ml. of a suitable dilution of the lysin were mixed with a small volume of bacterial cells so that the mixture contained a known initial concentration, generally 6 to 10×10^8 bacteria per ml. For each test, all samples received a constant, initial amount of cells (unless otherwise stated). The samples were incubated at 37°C. and readings were made at intervals. The changes in optical density were converted to changes in bacterial count by the use of standard curves prepared from direct microscopic counts of *S. aureus* K_1. The amount of bacterial debris remaining after lysis did not interfere with the estimates of cells lysed.

From the data, the course of lysis could be represented by plotting the logarithm of the unlysed bacteria against time at any given temperature, etc.

Estimates of lysin activity were based upon determinations of the initial velocity constants, K, calculated from the equation

$$K = \frac{2.3}{t} \log \frac{B_o}{B_o - B_t},$$

in which B_o represents the initial concentration of cells per ml., B_t are the cells per milliliter lysed in any given time interval, t. These "constants" are reported only for purposes of comparison within an individual experiment.

Less accurate estimates of lysin activity were made by determining the per cent bacteria lysed in a short time interval, and comparing the per cent lysis instead of the velocity constants, K, for different samples.

Procedure and Results

It has been found that neither virolysin nor autolysin affects freshly harvested, undamaged cells; both lysins, however, cause lysis after cells have been "sensitized" by a variety of damaging treatments: heat (from 45–120°C.), acetone, detergents, ultraviolet irradiation, penicillin. Phage also causes sensitization, and a combination of phage and lysin added to living cells initiates a lysis resembling typical lysis-from-without (Ralston *et al.*, 1955).

In this paper, the comparative study of the two lysins has been based mainly on their action on cells killed by heat. Identical results were obtained with cells killed at 56°, 100°C., or autoclaved for 20 minutes at 15 pounds per square inch. This method of sensitization was used to destroy intracellular enzymes. Unless otherwise noted, the experiments were performed with the 14 (K_1) virolysin and the K_1 autolysin.

I. Source of Virolysin and Autolysin

A. Preparation of Virolysin.—Virolysin is obtained from lysates of phage-infected cells. Phage lysates are prepared by inoculating double strength tryptose-phosphate broth with an 18 hour agar-grown *S. aureus* K_1 suspension at a concentration of 1×10^8 cells per ml. and phage 14 at 1×10^6 particles per ml. The cell-phage mixture is shaken at 37°C. until lysis occurs (usually 3 hours). The lysate contains both virolysin and phage (*ca.* 10^{10} particles per ml.).

This method of virolysin preparation involves several rounds of infection, since the cells outnumber the phage particles by 100 to 1. Fig. 1 shows the appearance of lysin and phage, as well as the changes in bacterial numbers during the process. Appreciable quantities of virolysin and phage appear at the time of mass lysis.

Lysates containing virolysin can also be obtained from cell suspensions undergoing a single cycle of infection, by adding 3 to 5 particles per cell to 1×10^8 bacteria per ml. under conditions similar to those described above. Lysis occurs at the end of a 40 minute latent period, and the release of virolysin and phage from the cell coincides with lysis. The appearance and increase in virolysin inside the infected cell during the latent period are described elsewhere (Ralston *et al.*, 1955; Ralston *et al.*, 1957 *b*).

Virolysin is separated from the heavier phage particles in the lysates by

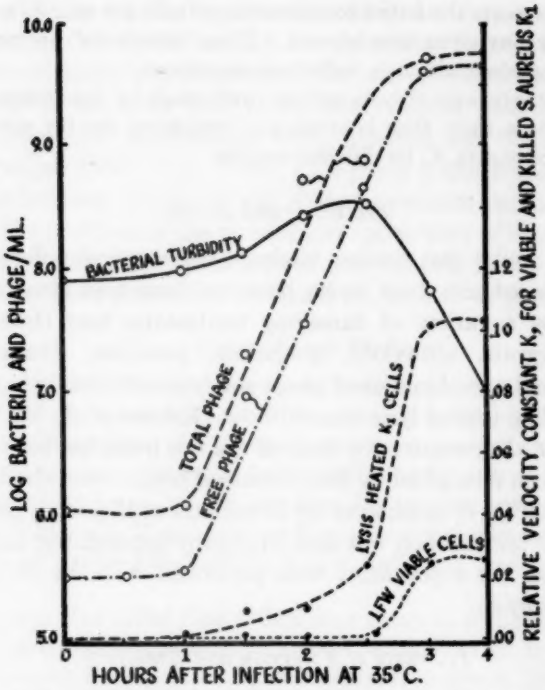

FIG. 1. Production of phage and virolysin in double strength tryptose phosphate broth at 37°C., when the initial phage/bacterium ratio was 1/100. The cell-phage mixture was removed at intervals and chilled in an ice bath. Total and free phage was determined by plaque counts of super-cel filtered and unfiltered samples. Virolysin activity was determined by lysis tests of samples which had been centrifuged to spin out the residual cells. Undiluted samples of the supernatant layer were mixed with 8×10^8 heat-killed *S. aureus* K_1 cells per ml. and incubated at 37°C. The velocity constant, *K* per minute, was calculated from the turbidimetric measurements of bacterial lysis. Undiluted samples of the supernatant layer were also mixed with 8×10^8 living K_1 cells per ml. and the velocity constant, *K* per minute, was calculated for their lysis at 37°C., as represented by the curve marked *LFW*. The lysis of living cells depends upon the presence of sufficient quantities of free phage to sensitize and of virolysin to lyse the cells. This lysis is considered to be a case of "lysis-from-without" (*LFW*).

spinning the lysate at 30,000 × *g* for 45 minutes at 6°C. in a Spinco model L ultracentrifuge. After 2 to 3 cycles of centrifugation, the phage in the supernate is reduced to 10^6 to 10^7 particles per ml. The lysin in the supernate can be precipitated by 40 per cent saturation with $(NH_4)_2SO_4$ at 4°C. The precipitate is then resuspended in mineral salts broth.

Virolysin can also be prepared by infecting the K_1 strain with phage 1 or the 145 strain of *S. aureus* with phage 1 or 14.

B. Preparation of Autolysin.—Autolysin is prepared from autolysates of uninfected cells of the K_1 strain grown in tryptose phosphate broth. Cells, grown on tryptose phosphate agar for 18 hours at 37°C., are inoculated into broth to a concentration of 1×10^8 bacteria per ml. and shaken at 37°C. for 6 hours. There is an increase in cell numbers, beginning at the 1st hour, from 1.0×10^8 cells per ml. to 2.5×10^9 cells per ml. at the 4th hour. The cell numbers then remain unchanged. At the 6th hour, the culture is removed from the shaker and stored at 4°C. for 1 to 5 days. During the storage period, autolysis occurs and, with it, the release of active autolysin.

Autolysis occurs much more rapidly with 3 hour cells stored at 4°C. or with 6 hour cells, layered with toluene and allowed to remain at 37°C.; however, under these conditions, either no autolysin is released, or, if present, it is very unstable. Agar-grown cultures autolyze very poorly and release little autolysin.

The autolysates are very viscous. Centrifugation of the autolysin at 30,000 $\times g$ for 45 minutes at 6°C. in the model L Spinco ultracentrifuge separates the lysin in the supernatant layer from the viscous material in the pellet. The lysin in the supernate can be precipitated with $(NH_4)_2SO_4$ at 40 per cent saturation at 4°C., and the precipitate resuspended in mineral salts broth.

C. "Crude" and "Semipurified" Lysins.—The ultracentrifuged supernates of the phage lysate or autolysate are referred to as "crude" lysins. The resuspended material from $(NH_4)_2SO_4$ precipitates is called "semipurified" lysin. Unless the term semipurified lysin is used, the experimental findings presented apply to the crude lysin.

Both lysins are adsorbed by a number of materials, including super-cel, alumina, charcoal; both are destroyed by the proteolytic enzymes, trypsin, chymotrypsin, and papain, but are not affected by ribonuclease or desoxyribonuclease.

II. Quantitative Aspects of the Lysis of Heat-Killed Cells by Autolysin and Virolysin

A. Effect of Cell (Substrate) Concentration.—Constant amounts of each crude lysin were mixed with varying amounts of cells. With both lysins, the curves representing the logarithms of the surviving bacteria per milliliter plotted against time, for each substrate concentration, showed an initial rapid decrease, followed by a slower lysis and an eventual end-point in the presence of considerable numbers (*ca.* 50 per cent) of unlysed cells. Microscopic study has shown the residual cells to be Gram-negative. They are resistant to fresh additions of either lysin and also to lysozyme, but are susceptible to trypsin.

When the data are corrected for these resistant cells, the course of lysis is

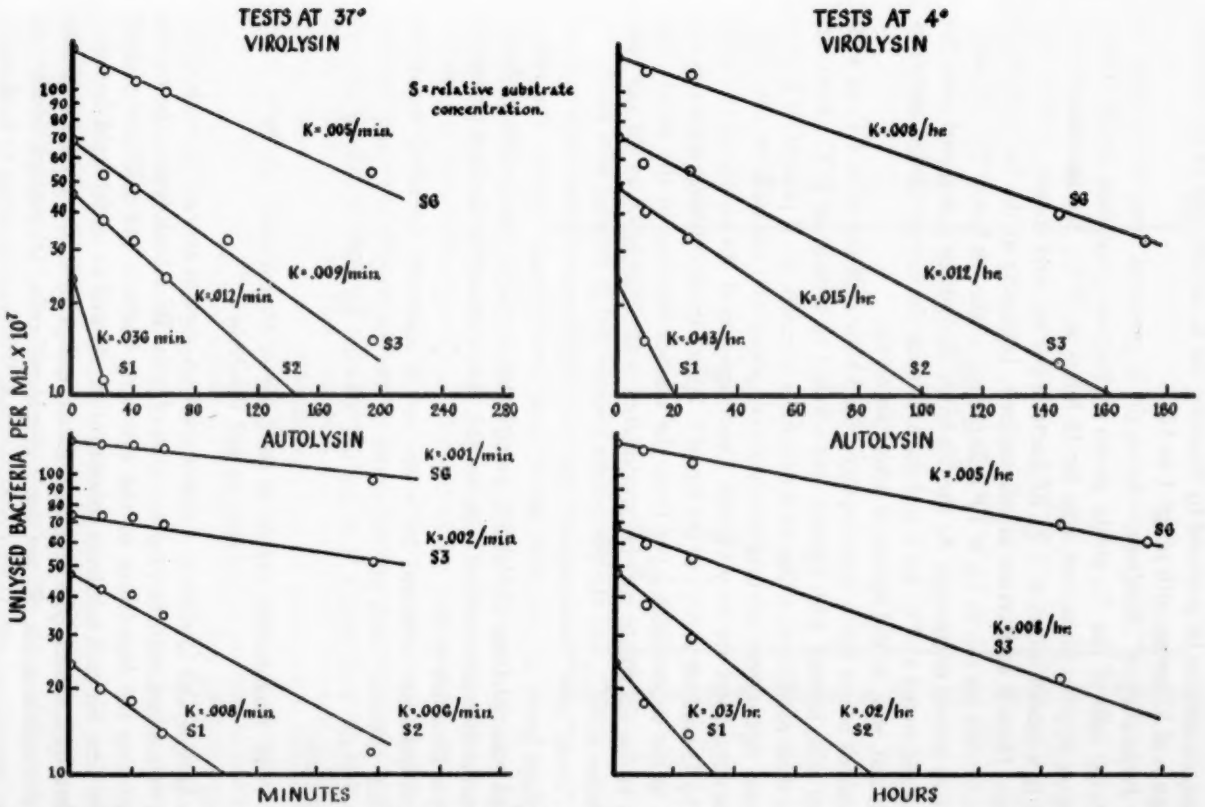

FIG. 2. Effect of substrate concentration (heat-killed K_1 cells) on lysis by constant amounts of autolysin and virolysin at their pH optima. (Data corrected for lysis-resistant cells.)

found to proceed logarithmically. With both lysins, the velocity constants, K, calculated from these curves, are decreased by raising the initial substrate concentration (Fig. 2).

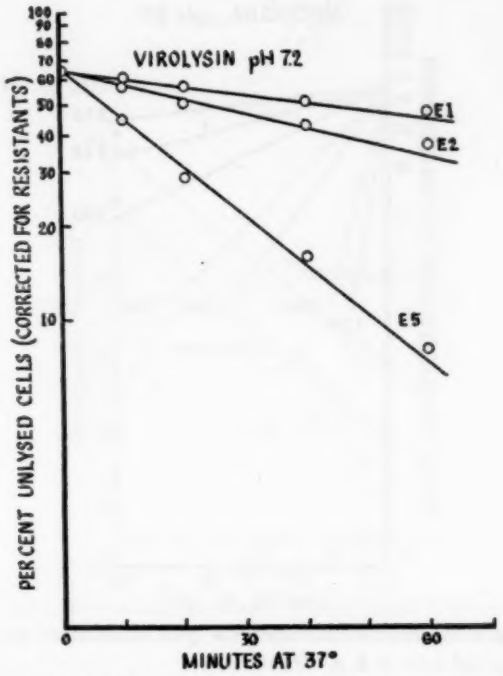

FIG. 3. Effect of virolysin concentration on lysis of constant amounts of heated K_1 cells at 37°C. Initial cells = 7×10^8/ml.

Relative lysin concentration (E)	Velocity constant (K)	$\frac{(K)}{(E)}$
1	0.005	0.005
2	0.010	0.005
5	0.038	0.007

B. Effect of Lysin Concentration.—Varying dilutions of each crude lysin were mixed with constant numbers of heat-killed cells at the pH optimum of each lysin. At all concentrations of the lysin, lysis came to an end-point in the presence of a constant number (*ca.* 50 per cent) of unlysed cells. Velocity constants, K, calculated from these curves (corrected for the resistant cells) showed a direct relationship to lysin concentration (Figs. 3 and 4).

C. Effect of Temperature.—Constant amounts of each crude lysin were added to constant amounts of cells at the pH optimum of each lysin and at tem-

at their pH optima. (Data corrected for lysis-resistant cells.)

peratures of 0, 28, and 37°C. With both lysins, lysis proceeds in the cold; the rate increases with rise in temperature. Velocity constants were calculated for the straight line portion of each curve, and these figures were used for deter-

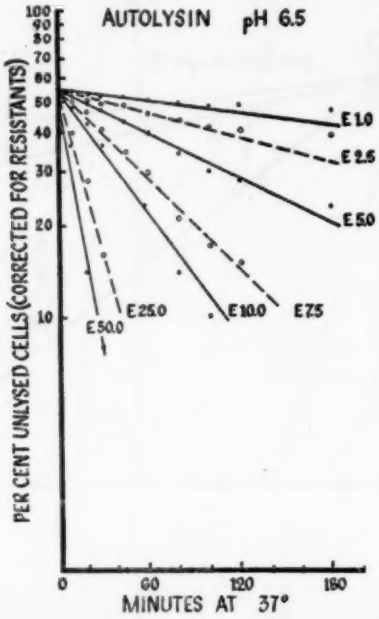

FIG. 4. Effect of autolysin concentration on lysis of constant amounts of heated K_1 cells at 37°C. Initial cells = 8×10^8/ml.

Relative lysin concentration (E)	Velocity constant (K/min.)	$\frac{(K)}{(E)}$
1	0.0018	0.0018
2.5	0.0031	0.0012
5	0.0067	0.0013
7.5	0.011	0.0015
10	0.016	0.0016
25	0.038	0.0015
50	0.069	0.0014

mining the temperature coefficients. The values for K and Q_{10} are given in Table I. Fig. 5 shows the logarithms of the velocity constants, K, plotted against the reciprocals of the absolute temperature for each case. Under optimal conditions of pH, the points for the three temperatures are not far from a straight line, suggesting that a single fundamental reaction determines the rate of lysis by virolysin and that a similar—but not necessarily identical reaction—

controls the rate of lysis by autolysin over the range tested. The values of μ, the critical thermal increment, calculated from the van't Hoff-Arrhenius equation,

$$K_2 = K_1 e^{\mu/2} \frac{(T_2 - T_1)}{T_2 T_1},$$

range from 11,800 to 20,000.

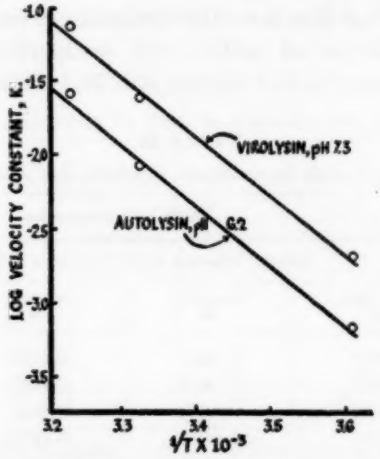

FIG. 5. Logarithms of velocity constants plotted against reciprocals of absolute temperature for lysis of *S. aureus* by virolysin and autolysin at their pH optima over the range 0–37°C.

TABLE I

Values of Velocity Constants and Temperature Coefficients for Lysis of Heat-Killed Staphylococci by Virolysin and Autolysin

Lysin	pH	$K \times 10^{-4}$, min.			Q_{10}	
		0°C.	28°C.	37°C.	0–28	28–37
Virolysin	7.3	0.288	3.0	5.32	4.3	1.9
Autolysin	6.2	0.047	0.77	2.03	6.8	2.9

III. Properties of the Lysins

A. pH Optima.—Differences in the pH optima of both crude and semi-purified lysins were shown by the following experiment: Lysis by autolysin and virolysin was determined over a wide pH range, both when the two agents were mixed together and when tested individually. Autolysin showed its maximum activity between pH 6.0 and 6.5 and virolysin between pH 7.0 and

7.5. The velocity constants for the lysis brought about by the mixture of the two lysins at each pH value (over the range pH 5.5 to 7.5) were found to equal the sum of the *K* values for each tested individually (Table II), indicating that the effects of mixing the two were additive, and that neither preparation contained any materials which might have affected the pH optimum of the other. Therefore, the two pH optima result from intrinsic differences between the two lysins.

B. Antigenic Relationships.—*(1) Antiserum to autolysin* inhibits lysis of killed K_1 cells by autolysin, but does not affect the action of virolysin. The antiserum was prepared by injection of rabbits with semipurified K_1 autolysin. The antiserum inhibited autolysin at a dilution of 1/50, but did not inhibit virolysin,

TABLE II

Lysis of Heat-Killed K_1 Cells by Autolysin, Virolysin, and Their Mixture at 37°C.

pH tested	Crude lysin preparation			
	Velocity constant, *K*/min., for lysis of K_1 cells by			
	Autolysin A	Virolysin B	Mixture M	Theoretical *K*, if M = A + B
5.5	0.0017	0.0017	0.0037	0.0034
6.5	0.0034	0.0028	0.0063	0.0062
7.5	0.0011	0.0040	0.0047	0.0051
8.5	0.0000	0.0020	0.0034	0.0020*

Active autolysin and virolysin mixed in equal parts at room temperature. Aliquots adjusted to various pH values with 1 N HCl or NaOH. Autolysin diluted 1:2 in boiled virolysin and virolysin diluted 1:2 in boiled autolysin for tests of individual lysin activity. Cell controls contained both inactive lysins (boiled) at the desired pH. Readings made at intervals by Klett turbidimetric method. Data were corrected for the per cent resistant cells.

* At pH 8.5, M varies somewhat above and below A + B from experiment to experiment.

even at 1/10. When autolysin and virolysin were mixed and treated with antiserum, the residual activity of the mixture equalled the activity of the virolysin alone, indicating that the virolysin preparation contained neither antiserum-sensitive nor antiserum-inactivating materials (Table III).

Table III shows that the antiserum also inhibits lysis-from-without by the combined action of autolysin and phage, but fails to inhibit lysis-from-without by virolysin and phage, indicating again that the antiserum is specific for autolysin.

The antiserum to K_1 autolysin has been found to inhibit the autolysin from *S. aureus* 145, but it has no effect on the virolysin 14(145) from this strain (table III).

(2) Antiserum to Phage P_1,—prepared by injection of rabbits with ultracentrifuged pellets containing phage, causes strong inactivation of P_{14} phage

but fails to inhibit either autolysin or virolysin at 1/200, a concentration which inactivated 10^8 phage particles per ml. in 20 minutes at 20°C.

These tests show that the two lysins are antigenically different from each other and that both are different from the phage P_1 (antigenically identical with P_{14}).

C. Effect of Chemical Inhibitors.—Both lysins are inhibited by the chemicals listed in Table IV. When cysteine is added to virolysin or autolysin which has been inhibited by compounds of heavy metals, activity is restored, suggesting that functional SH groups may be involved (Sumner and Somers, 1947).

TABLE III

Effect of Antiserum to K_1 Autolysin on Lysis by Autolysin and Virolysin Lysin Activity

Lysin	A Per cent lysis heat-killed K_1 cells by		B Per cent lysis heat-killed 145 cells by		C Per cent lysis phage-sensitized K_1 cells by	
	Untreated lysin	Neutralized lysin	Untreated lysin	Neutralized lysin	Untreated lysin	Neutralized lysin
K_1 autolysin	16	0	—	—	32	8
14(K_1) virolysin	23	22	—	—	63	67
Mixture A + V	43	27	—	—	—	—
145 autolysin	29	2	32	3	—	—
14(145) virolysin	47	45	51	51	—	—

For neutralization, lysins incubated with 1/25 antiserum or normal serum, for 2 hours at 20°C. Residual lysin tested by adding 6×10^8 cells per ml., incubating 30 minutes at 37°C., and calculating per cent lysis by turbidimetric readings.

Reinvestigation of our earlier results with iodoacetate disclosed that whereas concentrations in the order of M/1000 were not inhibitory, greater amounts, in the order of M/100 were effective.

Quantitative differences were observed in concentrations of certain inhibitors required. The activity of crude virolysin was depressed by M/5000 $PbCl_2$; that of crude autolysin was hardly affected. With Na citrate, M/100 caused inhibition of crude autolysin but not of virolysin. These differences may have been due to impurities in the crude lysin preparations and to the influence of pH on the inhibition, since each test was carried out at the pH optimum of the lysin.

Na desoxycholate had no inhibitory action on the crude lysins, but it depressed the activity of their semipurified preparations.

D. Effect of Versene on Virolysin and Autolysin.—The addition of 0.02 per cent versene (disodium salt of ethylenediaminetetraacetic acid) to either lysin causes strong inhibition of lysis of heat-killed K_1 cells. Various metallic cations are able to reverse this inhibition: M/1000 Ca^{++}, Mn^{++}, Co^{++}, and Zn^{++}. Mg^{++} is effective only for virolysin at M/1000, and concentrations 20-fold greater failed to reverse inhibition of autolysin (Table V).

TABLE IV

Inhibitors of Virolysin and Autolysin Activity for Heat-Killed S. Aureus K_1 and M. Lysodeikticus

Inhibitor	Final concentration	Effect on virolysin Per cent inhibition of lysis	Effect on autolysin Per cent inhibition of lysis	
		S. aureus K_1	*S. aureus* K_1	*M. lysodeikticus*
$CuSO_4$	M/1000	83	27	13
"	M/200	—	100	100
Merthiolate	0.1 per cent	45	29	51
"	0.2 per cent	—	59	39
Iodoacetate	M/1000	21	3	0
"	M/100	93	61*	—
Formalin	0.1 per cent	93	82	17
"	0.4 per cent	—	100	70
$AgNO_3$	M/1000	97	95	100
$HgCl_2$	M/1000	100	29	23
Duponol WA	0.1 per cent	100	82	60
Na-citrate	M/100	7	56	26
" "	M/20	83	80	48
I_2 in KI	M/1000	100*	79*	—
$K_4Fe(CN)_6$	M/300	100*	100*	—
$PbCl_2$	M/5000	53*	15*	17*
"	M/2500	47*	50*	—
Na-desoxycholate	0.2 per cent	80*	86*	—

* Experiment conducted with semipurified lysin; otherwise, tests run with crude lysins.

Lysin and inhibitor were mixed with constant numbers of test cells, 8×10^8 cells per ml. in tryptose phosphate broth. Samples were incubated at 37°C. After a constant time, dependent upon the activity of the untreated lysin control, the number of bacteria lysed was estimated from the turbidimetric readings. The per cent inhibition of lysis was calculated from:

$$1.0 - \frac{\text{per cent lysis with inhibitor}}{\text{per cent lysis without inhibitor}} \times 100$$

The following agents failed to inhibit autolysin and virolysin: M/1000 semicarbazide, Na-azide, Na-arsenite, sulfanilic acid, ascorbic acid, Na-fluoride, thiourea, K-oxalate, Na-sulfite, Na-tetraborate; 0.0025 per cent proflavin; 0.1 per cent pectin, 0.001 per cent acriflavin; 0.1 per cent saponin; 20 per cent toluene, and chloroform.

E. Thermolability.—Crude virolysin and crude autolysin are completely inactivated by boiling for 5 minutes. Crude virolysin is unstable even at 37°C., whereas crude autolysin is not (Table VIA), possibly because of certain protective substances in crude autolysin preparations. These substances reduce the heat inactivation of crude virolysin at 37°C., when the two crude lysins are mixed together. These materials in the crude autolysin are at least partially removed by precipitation of the lysin with $(NH_4)_2SO_4$.

TABLE V

Versene Inhibition of Autolysin and Virolysin and Its Reversal by Divalent Cations

Sample contents	Virolysin, pH 7.3		Autolysin, pH 6.5
	Per cent lysis heat-killed K_1 after 60 min., 37°C.	Per cent lysis heat-killed K_1 after 60 min., 37°C.; overnight at 4°C.	Per cent lysis heat-killed *lysodeikticus* 180 min., 37°C.; and overnight at 4°C.
Lysin control L	55	41	33
Lysin, L, + versene 0.02 per cent, V	12	13	11
L + V + Mg^{++} M/50	30	12	6
L + V + Mg^{++} M/1000	31	12	19
L + V + Mn^{++} M/1000	40	49	43
L + V + Ca^{++} M/1000	45	43	40
L + V + Co^{++} M/1000	39	40	32
L + V + Zn^{++} M/1000	26	42	30

Aliquots of lysin, diluted in double strength tryptose phosphate broth, exposed to versene, inorganic salt, or equivalent volumes of distilled water. Cells added to 8×10^8 bacteria per ml.

Both semipurified lysins are thermolabile. The inactivation of semipurified virolysin at 37°C. and of semipurified autolysin at 37° and 45°C. is given in Tables VIB and VIII. While the semipurified autolysin may be more heat-resistant than the semipurified virolysin, this may be the result of incomplete removal of the non-specific protective substances in the crude autolysin.

F. Specificity: Spectrum of Bacteria Lysed by Autolysin and Virolysin.—*(1) S. aureus* K_1.—With both lysins, heat-killed logarithmic phase cells are more sensitive than heat-killed resting cells. Acetone treatment has often increased the sensitivity of heated resting cells to virolysin but not to autolysin.

(2) Heterologous S. aureus Strains.—Both lysins act on all heat-killed *aureus* strains (25) tested so far. The ratio of their relative activities, however, varies from strain to strain.

(3) Heterologous Micrococcus Species.—Heat-killed cells of the following species are resistant to both lysins: *M. tetragenes*, *M. roseus*, *M. citreus*, *M. agilis*, *Sarcina sp.* Heated cells of *M. lysodeikticus* are relatively resistant to virolysin but very susceptible to autolysin; the degree of susceptibility apparently depends on cell age and method of subsequent treatment (Table VII).

TABLE VI

Heat Inactivation of Crude and Semipurified Lysins at 37°C.

	pH at which sample was heated at 37°C. and tested	Time at 37°C.	Virolysin-autolysin mixture			Theoretical if $M_{hrs.} = \frac{1}{2}V_{hrs.} + A_{hrs.}$
			Residual lysin *K* per min.			
		hrs.				
A. Crude lysins	6.0	0	0.008	0.007	0.020	
		2	0.002	0.005	0.013	0.013
	6.8	0	0.022	0.005	0.033	
		2	0.008	0.009	0.029	0.031
	7.6	0	0.016	0.002	0.020	
		2	0.009	0.004	0.017	0.020
B. Semipurified lysins	6.3	0	0.002	0.027		
		2	0.000	0.013		
	6.8	0	0.006	0.020		
		2	0.000	0.016		
	7.3	0	0.007	0.014		
		2	0.000	0.009		
	7.8	0	0.006	0.005		
		2	0.000	0.008		

In A, crude autolysin was diluted in boiled virolysin, virolysin diluted in boiled autolysin for tests of individual lysin heat lability. The active lysins were diluted in each other for tests of heat lability for mixture. Samples adjusted to desired pH with 1 N NaOH or HCl, heated for 2 hours, and residual lysin was determined by turbidimetric measurement of lysis of 8×10^8 bacteria per ml. of heated K_1 cells at 37°C. *K* per minute calculated from data corrected for 50 per cent lysis-resistant cells.

In B, same procedure used for semipurified lysins (the semipurified lysins were not prepared from crude lysins used in A). Mixture of the 2 semipurified lysins showed less heat protection of virolysin by autolysin.

TABLE VII

Effect of K_1 Autolysin, 14(K_1) Virolysin, and Their Mixture on Lysis of Acetone-Killed M. lysodeikticus at pH 6.2–8.0 at 4°C.

pH	Relative lysin activity per cent lysis			
	Experimental			Theoretical
	V	A	A + V	A + V
6.2	0	34	34	34
6.6	1	40	37	41
7.0	0	23	22	23
7.4	2	24	29	26

V = virolysin, A = autolysin, A + V = mixture.

Autolysin and virolysin were diluted one-half in each other or in boiled (inactive) lysin. Cells of acetone-extracted *M. lysodeikticus* were added to a final concentration of 8×10^8 bacteria per ml., after adjusting pH with NaOH or HCl. The per cent lysis was determined from turbidimetric readings after 2 days at 4°C. The concentrations of virolysin and autolysin used in the test were sufficient to cause extensive lysis of heat-killed K_1 cells.

Conceivably the strong activity of autolysin for *M. lysodeikticus* could be due to a contaminating lysin present in autolysin preparations but absent from virolysin preparations. This is not probable, because (1) autolysin preparations show the same pH optimum for K_1 and *M. lysodeikticus*, (2) the same chemical inhibitors prevent the lysis of both hosts by autolysin preparations, (3) heating

TABLE VIII

Inactivation at 45°C. of Autolysin Activity for Killed K_1 and M. lysodeikticus

Time heated at 45°C.	Residual activity, per cent lysis of 8×10^8 bacteria per ml. after 1 hr., 37°C., in samples heated and then tested at pH									
	5.8		6.3		6.8		7.3		7.8	
	Lys.	K_1	*Lys.*	K_1	*Lys.*	K_1	*Lys.*	K_1	*Lys.*	K_1
min.										
0	25	15	30	34	29	28	14	15	7	14
15	25	21	24	28	23	20	8	8	7	8
30	20	20	22	21	21	15	9	4	5	2
60	17	13	21	25	17	15	3	0	3	6

Logarithmic phase K_1 and *M. lysodeikticus* cells (*Lys.*) heat-killed for 60 and 30 minutes respectively just prior to test. Autolysin preparation: semipurified lysin at pH 6.5 and adjusted aliquots with 1 N NaOH and HCl. Heated at 45°C. and tested at same pH.

at 45°C. destroys the lytic activity in autolysin preparations at comparable rates for both hosts (Tables IV and VIII).

A second possible reason for the observed difference in activity could have been that virolysin preparations contained an inhibitor which prevented its action on *M. lysodeikticus*. This was not so, as indicated by testing mixtures of the two lysins over a pH range (Table VII). With *lysodeikticus* cells resistant to virolysin, the lysis in the mixture of the two lysins equalled that caused by autolysin alone. The results are best explained by assuming that the two lysins show different substrate specificities.

4. Heterologous Genera.—Simultaneous comparisons of both autolysin and virolysin have shown the following to be resistant: *Escherichia coli*, *Serratia marcescens*, *Bacillus mycoides*, *Bacillus subtilis*.

The following have been found to be resistant to virolysin and were not tested with autolysin: *Azotobacter chroococcum*, *Streptococcus species*, *Neisseria perflava*, *Propionibacterium jensenii*, *Corynebacterium xerose*, *Brucella melitensis*, *Listeria monocytogenes*, *Rhodospirillum rubrum*, *Caulobacter species*, *Streptomyces species*, *Mycobacterium tuberculosis*, *Cytophaga johnsonae*, *Anabaena cylindrica*.

Certain pseudomonads underwent slight but reproducible lysis with both lysins: *Pseudomonas fluorescens*, *Ps. aeruginosa*, *Ps. species*. It is uncertain whether this effect is caused by autolysin and virolysin, since no tests were made to rule out the possibility that a contaminating lysin could be responsible.

DISCUSSION

Both virolysin and autolysin produce lysis of cells which have been boiled for several hours, a procedure which destroys the intracellular enzymes of the bacterium. It is assumed, therefore, that no bacterial enzymes are involved in the action of either lysin and that the lysins act directly upon a heat-stable component of the cell. Both lysins have many of the properties associated with enzymes. Each is thermolabile, is destroyed by proteolytic enzymes, has a pH optimum, has substrate specificity, is inhibited by chemicals known to be "enzyme inhibitors," etc. With respect to the influence of temperature, lysis has the characteristics of a chemical reaction.

These findings suggest that cell lysis results from a chemical reaction involving a bacterial substrate and that this reaction is catalyzed by the enzyme virolysin or autolysin. The substrate may be a polysaccharide (Lieberman, 1956; Lieberman and Krueger, to be published) and probably forms the framework of the cell wall.

Since the lysins cannot act on cells unless they have been previously damaged by a "sensitizing" agent, it appears that a protective mechanism normally exists to prevent either lysin from acting on the substrate.

The lysin produced by the phage-infected cell is antigenically distinct from the lysin of the uninfected cell; this implies that the phage-synthesizing apparatus is not necessarily restricted to the use of preformed bacterial enzymes but rather can induce the appearance of an enzyme which is not present in detectable quantity in the normal cell.

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THE PROTEIN COATS OR "GHOSTS" OF COLIPHAGE T2

I. Preparation, Assay, and Some Chemical Properties

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(Received for publication, November 5, 1956)

With the discovery (1) that the protein coat or "ghost" of coli bacteriophage T2 carried some of the biological functions of the parent virus, it was possible to examine more carefully than heretofore the relationship of biological function to morphological structure and chemical constitution. In the series of papers to follow data will be given to support the statements in the original report (1) and to demonstrate that many properties usually associated with the intact virus are found in the protein component, suggesting a high degree of specialization and organization in this relatively small biological unit.

In this, the first, paper will be described the preparation, fractionation, method of assay, and some physicochemical properties of the ghosts of T2 phage. The biological properties of the ghosts will be described in the second paper while studies of the metabolic changes induced in the host cell by ghosts (2, 3) will appear in the third and fourth papers. The inhibitory effect of divalent cations (4) on the action of ghosts will be presented in the fifth paper of this series.

In the interim since the first report a number of papers (5–14) have dealt to a varying degree with some of the properties of ghosts. These will be discussed in the text of this and succeeding papers.

Materials and Methods

Many of the materials and methods used in the present work are the usual ones (15, 16) described for the T phages of *Escherichia coli* B. The details are found either in the protocols or in the section on Experimental Details near the end of this paper.

Only highly purified phage (titer/E_{400} > 2) (17) was used in the present experiments. In most instances freshly shocked but unfractionated ghosts were used but in certain critical experiments the results were confirmed with ghosts freed of nucleic acid as described in Table I. These instances are noted in the protocols.

* Aided by a grant from the National Foundation for Infantile Paralysis.

‡ Supported in part by a grant from the Atomic Energy Commission to Dr. Herriott.

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TABLE I

Preparation and Purification of T2r+ Ghosts

T2r+ phage: titer 1.8×10^{13}/ml.; titer/$E_{400} = 2.7 \times 10^{12}$; mg. N/ml. = 1.5; P/N = 0.30.

Number		Volume	Ghosts/ml.*	Total ghosts	Total
					per cent
1	2.9 ml. of the above phage + 6 ml. 3 M Na_2SO_4 for 2 min., then + 140 ml. cold water rapidly with agitation Residual infectivity by plaque count was 5×10^8/ml.	149	3.5×10^{11}	5.2×10^{13}	100
2	Number 1 + 0.15 ml. saturated $MgSO_4$ (2.8 M) + 0.15 mg. DNAse left at 5°C. overnight	2	3.5×10^{11}	5.2×10^{13}	100
3	Number 2 centrifuged at 3500 for one half hr.; supernatant	149	3.5×10^{11}	5.2×10^{13}	100
4	Number 3 centrifuged at 100,000 × *g* for 1 hr. in a Spinco refrigerated centrifuge at 10°C.				
5	Supernatant from number 4	134	3.0×10^{10}	4.0×10^{12}	9
6	Residue from number 4 dissolved in cold saline	4	3.0×10^{12}	3.6×10^{13}	70
7	Number 6 centrifuged at 2,000 for 15 min.; supernatant	4	8.3×10^{12}	3.3×10^{13}	64
8	Number 7 centrifuged at 18,000 × *g* in a Servall SS-2 for 1 hr.				
9	Supernatant from number 8	3.8	3.8×10^{11}	1.4×10^{12}	3
10‡	Residue from number 8 dissolved in saline	4	4.8×10^{12}	3.1×10^{13}	60

* See Fig. 3 and the text for discussion of the lytic assay unit.

‡ Some chemical and physical properties of this and similar preparations are reported in Table IV.

EXPERIMENTAL

Preparation and Purification of T2 Ghosts.—Anderson (18) made the important discovery that rapid dilution of a strong salt solution of the T-even phages leads to a loss of infectivity and of the Tyndall scattering. After using this operation which Anderson termed "osmotic shock" to prepare undegraded

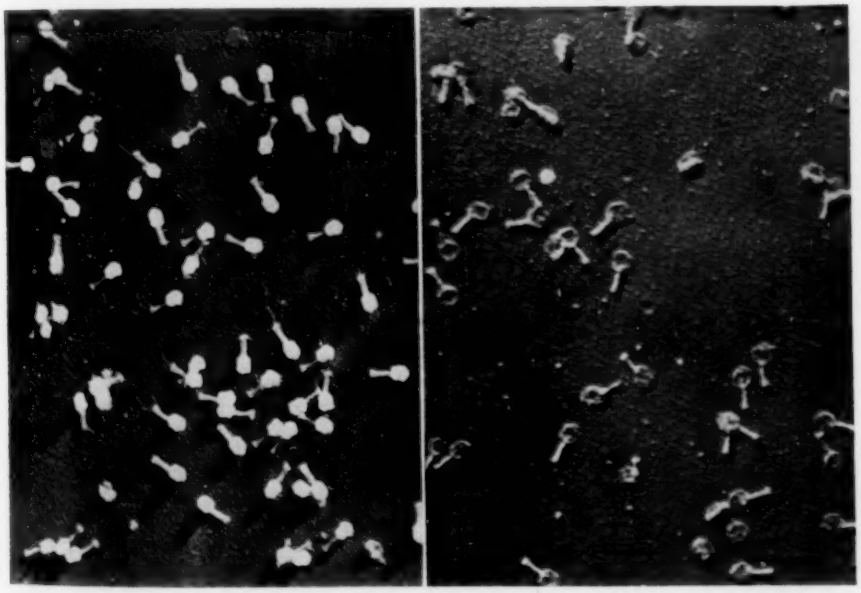
deoxyribosenucleic acid (DNA) it was observed (1) that the shocked phage lysed growing host cells much sooner than did an equivalent quantity of phage. Earlier, Anderson (19) had reported a "lysin" from heavily irradiated phage preparations, but it was not sedimented in an hour at 34,000 × *g* and its action was detected on *irradiated* host cells. Since in the present instance the lytic factor sediments and lyses normal host cells, it may be different although no direct comparison has been made.

Table I contains the results of an experiment in which the lytic factor in shocked phage was purified by differential centrifugation, after first degrading the nucleic acid with the enzyme, deoxyribonuclease (DNAse). From this and other comparable experiments it was observed that:

1. Osmotic shock destroyed 99 per cent or more of the plaque-forming capacity of a preparation, as had been noted earlier by Anderson (18).

2. The viscous phage DNA appeared in solution as a result of the shock treatment and it was digested with DNAse without any detectable effect on the lytic activity of the preparation. This has been confirmed recently by Bonifas and Kellenberger (11).

3. The lytic property sedimented and was recovered on resuspension of the residue.



(*a*) (*b*)

FIG. 1. Electron photomicrographs taken through the cooperation of Dr. James S. Murphy of: (*a*) T2 coliphage, and (*b*) the protein coats or ghosts prepared from the T2 phage by osmotic shock.

TABLE II

Phage Titer vs. Lytic Titer of Ghosts from Purified Phage

Sample	Method of converting to ghosts	Titer of parent phage		Lytic ghost titer from standard curve Fig. 3	Killing by an average of 3 ghosts per cell
		$1/E_{400}^{1\text{ cm.}}$	1/ml.		
					per cent
1	Na_2SO_4-osmotic shock	2.0	4.6×10^{13}	5×10^{13}/ml.	
2	" " "	2.3	4×10^{12}	4×10^{12}	
3	" " "	2.3	1.7×10^{13}	1.6×10^{13}	
4	" " "	2.7	1.8×10^{11}	1.7×10^{11}	
5	" " "	2.6	2.1×10^{13}	1.75×10^{13}	
6*	" " "	2.1	7×10^{12}	5×10^{12}	72
	Glycerin-osmotic shock	2.1	7×10^{12}	5×10^{12}	72
	Polyethylene glycol-osmotic shock			5×10^{12}	73
	Sucrose crenation	2.1	7×10^{12}	5×10^{12}	77
	0.01 M pyrophosphate pH 8.5	2.1	7×10^{12}	5×10^{12}	79

* This and one other sample which had been recently prepared and stored in 0.05 M NaCl, 10 μg. gelatin, and a drop of chloroform showed a decreased lytic ghost titer compared to its infectivity.

4. Through the kindness of Dr. James S. Murphy, a few electron microscope examinations showed that the number of ghosts (shown in Fig. 1 *b*) correlated in a rough manner with the lytic power of the solution. Thus, when sedimentation removed most of the lytic activity of the solution, it had removed most but not all the ghosts. When no lytic activity remained, no ghosts were seen on the electron micrographs.

5. Since the lytic activity could be sedimented in an hour at 18,000 $\times$ *g* it must be associated with a relatively large or dense structure.[1]

In Table II are the analyses of a number of preparations of ghosts. Many of them were prepared by the usual osmotic shock method, but for a few preparations other methods were used. These results show that the lytic property is relatively constant and is independent of the method of producing the ghosts. Thus far, attempts to dissociate the lytic activity from the ghosts have been unsuccessful. The conclusion is unavoidable, therefore, that the lytic activity of shocked T2 phage is closely associated with the protein coat or ghost.

Lytic Assay.—The curves in Fig. 2 illustrate how the turbidity of *E. coli* B suspensions is affected by the introduction of various quantities or concentrations of shocked phage over the course of 30 minutes. Since a large increase

[1] Ghosts do not sediment from the original "shockate" following DNase treatment as readily as they do after a preliminary high speed sedimentation and resuspension in saline.

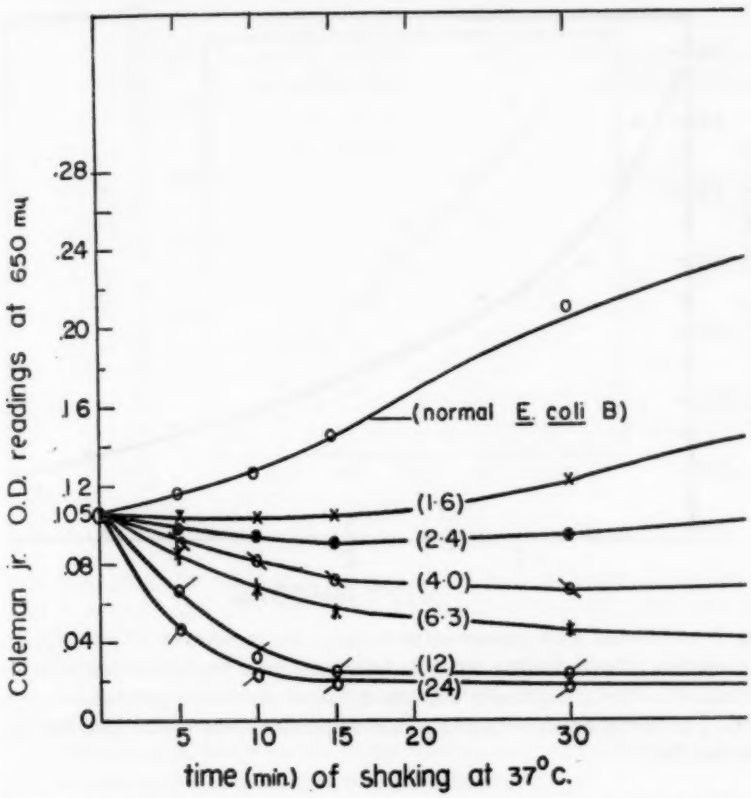


FIG. 2. Changes in turbidity with time of suspensions of nutrient broth-grown *E. coli* B following the addition of various quantities of T2 ghosts. The figures in parentheses represent the average number of ghosts per bacterial cell.

in phage takes only 25 minutes in broth, any evaluation of the ghosts must be made before this time.[2] The information in these curves was used to develop a convenient assay method for ghosts. The optical density values in Fig. 2 at 15 minutes are plotted in Fig. 3 against the corresponding number of ghosts in the 1 ml. that was added to 10 ml. of standard cell suspension. Since purified phage preparations were used in the preparation of the ghosts, the total number of ghosts was taken from the phage titer prior to shocking.

To analyze an unknown requires only the addition of 1 ml. of a saline dilution of a preparation containing 0.75 to 2.5 $\times$ 10^{10} ghosts/ml. to 10 ml. of 3.8 $\times$ 10^8 *E. coli* B per ml. in nutrient broth (O.D. = 0.115).[3] This 11 ml. suspen-

[2] The action of ghost preparations having too low a phage concentration to complicate the results is shown in Fig. 3 of the next paper (20).

[3] The optical density in this system equals 1.5 $\times$ $E_{1\text{ cm.}}^{650\ m\mu}$ or a Beckman reading of 0.077.

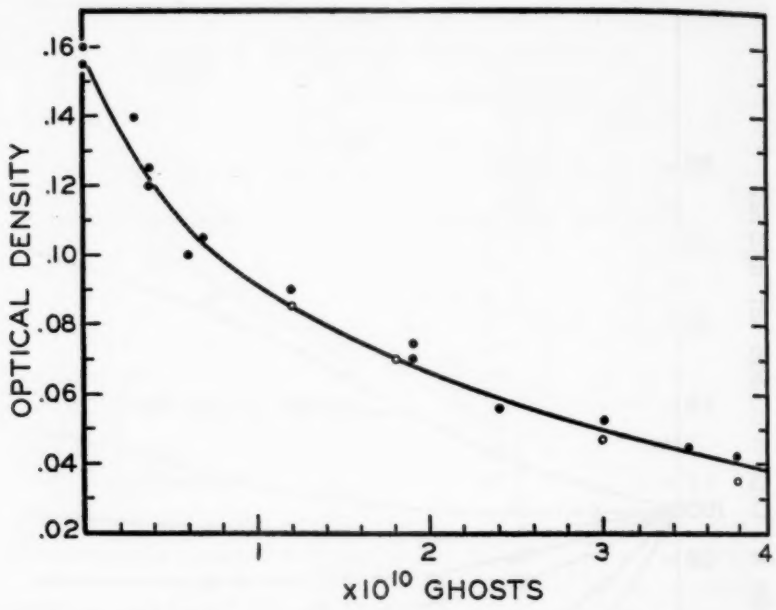


FIG. 3. A standard lysis curve used to evaluate the number of T2 ghosts in an unknown solution. The turbidities as optical densities were read after shaking mixtures of a standard bacterial suspension with the indicated number of ghosts for 15 minutes at 37°C. The initial density reading of the suspensions was 0.105. (See the text for further details.)

sion, which in a 22 x 175 mm. optically uniform test tube measures 0.105 O.D. units in a Coleman Jr. spectrophotometer at 650 mμ, is shaken at 37°C. for 15 minutes and read again along with a control suspension of uninfected cells. The density of the control tube indicates whether the culture has the growth properties of the system used in preparing the curve in Fig. 3. From the optical density readings of the experimental tube the number of ghosts per milliliter added may be found by reading off the curve in Fig. 3. In general, parallel analyses agree within ±15 per cent though values on different days occasionally differ by as much as ±25 per cent. Reproducible results are obtained when cells are grown up as needed from concentrations no higher than about 2×10^7/ml.; i.e., lag phase cells must be diluted below this figure and allowed to grow to 3.8×10^8/ml. (O.D. = 0.115). Mutants that appear to be more resistant to lysis by ghosts accumulate occasionally after numerous transfers of the stocks so that an occasional isolation of the more susceptible stock is recommended.

The results of analyses of different ghost samples are shown in Table II.

Proof of Lysis of Cells by Ghosts.—In the foregoing paragraphs it has been

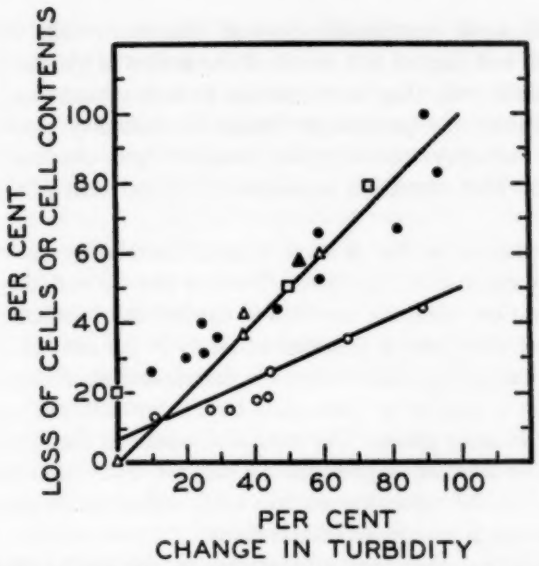


FIG. 4. Changes in turbidity as a measure of cell lysis by ghosts. Lysis has been estimated in several ways. The loss of 260 mμ absorbing material from cells grown in synthetic medium containing 1×10^{-4} M magnesium ion is denoted by the solid circles in the above figure. The open circles represent a similar measurement on cells grown in the presence of 2 to 5×10^{-3} M magnesium ion. The open triangles were obtained by direct microscopic count on cells grown in nutrient broth. The open squares derive from analyses of radioactivity released into the medium by the action of ghosts on broth-grown cells labelled with phosphorus 32.

assumed that changes in turbidity of the cell suspension were a reflection of changes in the number of cells and that a drop in turbidity could be taken as lysis of some cells. It is known (21), however, that changes in turbidity are not always accompanied by changes in number of cells, so that the action of ghosts on coli cells was examined in more detail to decide whether changes in turbidity are correctly a reflection of cell lysis.

Three criteria of lysis were chosen: (1) release of P^{32} from labelled cells; (2) microscopic count of residual cells; and (3) release of material absorbing at 260 mμ. In Fig. 4 are plotted the criteria listed above against the change in turbidity. The microscopic count and P^{32} release experiments were performed with the cells suspended in nutrient broth, the medium used in the assay procedure. For absorbancy measurements the broth could not be used and a synthetic medium (17) was substituted. Since the concentration of magnesium ion is higher in the synthetic medium than in broth, and the divalent cations affect the ghost action (4, 22), another set of experiments was performed in synthetic medium containing 1×10^{-4} molar magnesium ion.

A phase microscope examination showed that many cells were larger and round instead of rod-shaped as a result of the action of ghosts. Since they appeared to be whole cells, they were counted as such without any correction.

Before calculating the percentage change all turbidity readings were corrected for the turbidity remaining at complete lysis obtained by using an excess of ghosts. This correction amounted to 15 per cent of the original cell turbidity.

The results plotted in Fig. 4 show a good correlation between turbidity changes and the number of organisms. Hence, a drop in turbidity is a measure of cell disintegration when the reaction is carried out in nutrient broth. The data also suggest that there is a release of up to 20 per cent of the total phosphorus and absorbing material without a corresponding change in turbidity. This observation is similar to that made by Prater (23) using phage and by Puck and Lee (9) using ghosts. The results obtained in the synthetic medium having the higher level of magnesium ion suggest that about half of the 260 mμ absorbing material (probably nucleic acid) sediments in this system with the cell debris even in low gravitational fields.

It is concluded from the above studies that in nutrient broth and probably in a synthetic medium ghosts induce the lysis of cells and that the fall in turbidity is a measure of the cells lysed.

Efficiency of Adsorption of Ghosts.—Although it seemed obvious that if the ghosts induce lysis of the host cells, this effect is preceded by adsorption of the ghosts. Nevertheless, it was important to evaluate the efficiency of this adsorption. The rate of adsorption might have changed in either direction as a result of the change from phage to ghosts.

The efficiency of adsorption to host cells was determined through the use of isotopically labelled ghosts. The experiments are described in Table III, where it is readily seen that *as phage*, 95 per cent of 2, 5, and 8 particles per bacterium were adsorbed in 5 minutes at 37°C. *As ghosts*, however, the value was closer to 80 per cent and this figure did not reach 90 per cent with added time of adsorption. These results are comparable to those reported by Hershey (6, 12) and more recently by French and Siminovitch (14). Using the electron microscope Bonifas and Kellenberger (11) reported that nearly 99 per cent of the ghosts were removed by adsorption. This discrepancy in findings suggests that non-sedimentable ghost protein may be formed following the union of ghosts and cells.

No correction for the fraction of unadsorbed material in terms of ghosts has been made in the ghost titers recorded in this or the other papers of the series.

The Unit of Ghost Action.—Since, as will be demonstrated in the succeeding papers, ghosts have many biological functions, some measure or unit was sought which would be applicable to all functions. The unit selected was the *number* of ghost particles. It has been shown (24, 17) that for purified T2 phage the infectivity titer (plaque-forming units) closely approximates the

number of particles in a sample having a diameter of 50 to 100 mμ. Ghosts are prepared directly from phage and can be assayed without fractionation so that any particular ghost property can be correlated with the number of ghosts established from a phage titer. This merely carries the assumption that all or a constant fraction of the phage particles are converted to effective lytic units, which thus far appears to be correct though there will be some comments on this point a little later. To prepare a standard curve such as the lytic curves in Fig. 3, unfractionated ghosts prepared from a relatively pure and precisely titered phage preparation are used. This takes advantage of the relative precision (±15 per cent) of the phage assay.

The data in Table II indicate the correlation between the ghost lytic titers read from the curves in Fig. 3 and the original infectivity titers of the several parent phage preparations.

Basing the unit of action on the number of ghosts has great convenience and reproducibility, but it conveys a misleading impression. Without presenting the detailed evidence, for it is to be discussed in the next paper, there is evidence for heterogeneity in the ghost preparations. Thus, it has been observed in this and other (11, 14) laboratories that not all the ghosts can "kill" the host cells. With such heterogeneity in the ghost preparations, basing the unit of ghost action on the number would appear to be meaningless. However, thus far three different methods of preparing ghosts have yielded products indistinguishable in their lytic action (see Table II) and until some method permits fractionation of these preparations the present unit has sufficient utilitarian value to recommend its use. Besides, no alternative unit of action offers as much.

Antigenicity.—Since the ghost makes up the bulk of the protein part of the

TABLE III

Adsorption Efficiency of S^{35}-Labelled Ghosts

	Ghosts per cell (average)					
	2		5		8	
	C.P.M.	Per cent	C.P.M.	Per cent	C.P.M.	Per cent
Ghosts* alone	5,367	100	11,800	100	19,900	100
Ghost + cells‡; supernatant	1,100	20	2,380	20	3,920	20
Adsorbed to cells (by difference)	4,267	80	9,420	80	15,980	80
Phage alone	6,300	100	12,630	100	20,130	100
Phage + cells‡; supernatant	220	3.5	460	4	1,120	5.5
Adsorbed to cells (by difference)	6,080	96.5	12,170	96	19,010	94.5

* These ghosts were prepared by osmotic shock from 2 M Na_2SO_4 solution.

‡ 2 × 10^8/ml. log phase *E. coli* B in nutrient broth were incubated with the phage or ghosts for 5 minutes at 37°C., after which aliquots were chilled and sedimented at 5,000 × *g* for 10 minutes. Aliquots of the supernatant were analyzed for radioactive sulfur.

virus, it was expected that it would be antigenic and that antisera to it would cross-react to some degree with the intact virus. However, in view of the marked changes in antigenic nature which accompany relatively small changes in proteins (25) it was important to confirm our expectations. Hershey and Chase (6), Lanni and Lanni (7), and Nagano and Oda (8) have recently obtained evidence indicating that the ghosts carry the antigenicity of the virus. Before these publications appeared we had prepared rabbit antisera to: (*a*) purified T2 virus (17); (*b*) osmotically shocked T2 virus; (*c*) purified T2 ghosts containing less than 0.01 per cent of the total particles as active virus and no measurable quantity of nucleic acid; and (*d*) a dilute virus control corresponding to the 0.01 per cent active phage in (*c*). It was found that the antisera to the first three antigens were nearly the same in their quantitative neutralizing action against either the infectivity of the virus or the lytic property of the ghosts. Velocity constants (15) for the reaction between the antisera and phage or ghosts in saline were 1200, 650, 600, and 22 respectively for the antisera prepared from the materials in (*a*), (*b*), (*c*), and (*d*). Thus, in agreement with the results of others the ghost is responsible for most of the antigenicity of bacteriophage T2 as tested by the power of the antisera to neutralize phage infectivity.

General Chemical Properties

The physical and chemical properties of a number of preparations of purified ghosts corresponding to sample 10 of Table I have been evaluated and the results are shown in Table IV.

The ghost titer per milligram of nitrogen is a little over two times greater than the corresponding figure for the parent phage preparation. This is in agreement with the value (17) of the protein content of this phage (*ca.* 40 per cent).

TABLE IV

A Comparison of Physicochemical Properties of Ghosts and Phage

	Ghosts	Phage
Titer/E_{280}	1.8×10^{13}	1.7×10^{11}
Titer/mg. N	$2.8 \pm 0.2 \times 10^{13}$	1.2×10^{13}
R.S.*/10^{13} particles	1.6	11
R.S.*/E_{280}	3.5×10^{-19}	1.9×10^{-19}
Titer/E_{400}	1.7×10^{13}	2.7×10^{12}
E_{260}/E_{280}	0.92	1.4
P/N	0.01	0.32

* R.S. stands for "Rayleigh slope," the slope of a plot of optical density against the reciprocal of the fourth power of the wave length in centimeters. The measurements were obtained at wave lengths between 1,000 and 700 mμ in a Beckman spectrophotometer.

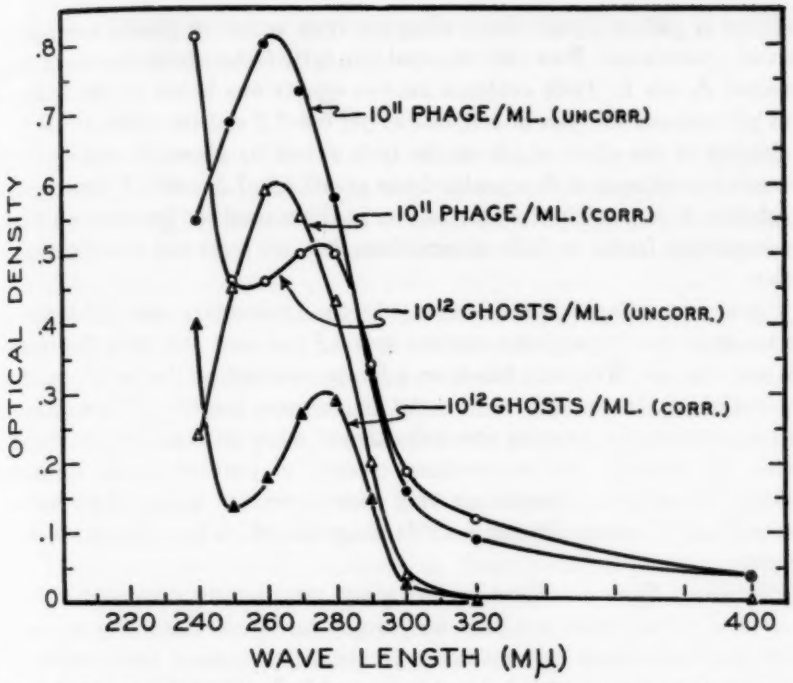


FIG. 5. Absorbancy curves of T2 phage and ghosts. See the text for a discussion of the correction for light scattering.

Absorption Spectrum.—Fig. 5 contains the absorption spectra of T2 phage and its purified ghosts both uncorrected and corrected for scattering. The scattering correction was made by assuming that the optical density value at 320 mμ represented the scattering at that wave length. The contribution of scattering to the values at the lower wave lengths was then calculated on the basis of Rayleigh's rule (35). According to this rule the scattering increases inversely as the fourth power of the wave length. Rayleigh's expression is applicable only when the particles are smaller than one-tenth of the wave length of the light used (35) and the particles in the present study are borderline with respect to this limitation; hence, it is only a first approximation. The absorption maximum for the ghost preparations was 275 to 280 mμ which is typical of proteins. Had there been appreciable nucleic acid present the minimum in the region of 250 mμ would not have been so low for the extinction coefficient of nucleic acids is many times greater than that of proteins. Since 1×10^{12} ghosts per ml. represents 33 μg. of nitrogen and approximately 230 μg. of protein, the corrected $E^{1mg./ml.}_{275m\mu} = 1.3$, and the comparable value for phage, $E^{1mg./ml.}_{262m\mu} = 13$. The corresponding uncorrected values are 2.2 and 17.5 respectively.

Effect of pH on Lysis.—Soon after the lytic action of ghosts was first observed, Quersin and Dirx (30) reported two lytic factors from lysates of phage infected *E. coli* B. Their evidence for two agents was based on the finding of two pH maxima for lysis of coli, one at pH 6.8–7.2 and the other at pH 8.

Studies of the effect of pH on the lysis of coli by ghosts in nutrient broth showed no evidence of two peaks. Lysis at pH 6.9, 7.3, and 7.7 were indistinguishable. It is possible that the lactate medium used by Quersin and Dirx is an important factor in their observations, but we have not investigated this point.

Tyrosine and Tryptophane Content.—In the preliminary note (1) it was reported that the tryptophane content was 3.5 per cent and that the tyrosine content was low. This was based on a faulty assumption that a failure of the absorption spectra to shift toward the longer wave length and to increase on making the solution alkaline was indicative of a low level of tyrosine. Table V shows the tyrosine and tryptophane content of purified ghosts by several methods of analysis. There is not very close agreement among them nor with the analyses of others (16, 12) for this phage for which no explanation can be offered.

Phosphorus Content.—Three or four ghost preparations comparable to fraction 10 of Table I were analyzed for phosphorus by the King (36) and/or the Fiske and SubbaRow (37) methods. In the latter method the samples after digestion were diluted with 1 ml. of water and boiled for 10 minutes to convert any pyrophosphate to orthophosphate (38). In all the preparations there was residual phosphorus content which relative to the nitrogen content was P/N

TABLE V

Tyrosine and Tryptophane in T2 Ghosts

Method	Per cent of protein	
	Tyrosine	Tryptophane
Microbiological*	4.3	0.9
Radioautograph‡	3–9	
Microbiological§	2.0	1.44
Colorimetric‖		2.0
"		1.5
Spectrophotometric¶	5.8	2.8
Colorimetric**	2.77	1.45

* From Luria's analyses (16).
‡ Hershey (12).
§ Following the method of Henderson and Snell (41).
‖ Following the method of Bates (42).
¶ Following the method of Beavan and Holiday (43).
** Reported by Cohen and Anderson (44).

= 0.01. Since the P/N ratio of T2 phage is about 0.3 (17) the ghosts have about 3 per cent of the original phosphorus.

DISCUSSION

There are many reports (26–34) of bacteriolytic agents produced by phage-infected cells, but in no instance has the evidence been such as to permit a clear understanding of the nature of the agent. In most instances the evidence excludes the whole phage and in some instances phage coats or ghosts from responsibility for the observed lytic activity. In the present instance there can be little doubt about the lytic agent. Highly purified phage was used as the starting point for the preparation of ghosts and all attempts to separate the lytic activity from the ghosts were unsuccessful. It is for this reason that the lytic activity is used as a measure of the number of ghosts. While this holds for coliphage T2 from *E. coli* B, the literature suggests that this would not be true for all systems.

It is perhaps worth making special note that although osmotic shock has been used in the preparation of most of the ghost preparations used in this work, other methods such as crenation or dilute pyrophosphate, two quite different methods, also produce ghosts by releasing the phage DNA. These ghosts are indistinguishable from those produced by osmotic shock.

It will have to remain for future studies to decide whether the route of nucleic acid release is the same in the above methods and whether the same as that utilized by the phage upon infecting its host. The released DNA appears to have no measurable effect on the ghost lytic property for there is no change during the action of DNAse. It is of some interest to recall that upon injection into the cell at the time of infection the injected material—largely DNA(12)—must set up some process inhibitory to lysis by the residual ghosts for multiply infected cells do not lyse rapidly unless the multiplicity is well over 50 or the energy metabolism is upset.

Other aspects of this discussion will be deferred to a succeeding paper.

Experimental Details

Preparation of Phage.—The methods of preparing, purifying (17), and titering (39) phage have already been discussed.

S^{35}-Labelled Phage.—The medium for the growth of labelled phage was the usual synthetic glucose-salts medium (17) except that the sulfate concentration was reduced to one-fifth the usual figure. When the cells had grown to 6×10^8/ml. and were infected with 1×10^6 T2r^+/ml., 5 millicuries of carrier-free S^{35} as sulfate was added per liter of medium. Growth and harvesting were similar to the procedure (17) previously described, except that precautions were taken to prevent excessive exposure of personnel to the radiation.

After purification the virus was found to contain 74×10^3 counts per minute per 10^{12} plaque-forming units.

P^{32}-Labelled Phage.—T2 phage labelled with 1000 C.P.M./10^{10} phage was prepared using the method described for unlabelled phage (17) with the slight modification of adding 20 microcuries of P^{32} phosphate ion per ml. of culture medium immediately after the cells were inoculated with phage.

Preparation of Ghosts.—

(*1*) *By Osmotic Shock.*—Most of the osmotic shock experiments were carried out by the method described in Table I. Sodium sulfate could be replaced by sodium acetate or ammonium sulfate but not by magnesium sulfate. In a few experiments non-ionic solutes such as glycerin and polyethylene glycol (a Carbide and Carbon Chemicals Co. preparation) were used in producing osmotic shock. Dilution with cold water gave better results as was noted by Anderson *et al.* (40). In our experience over 99 per cent of the particles were inactivated and sometimes it was 99.9 per cent. In one or two instances phage preparations were found to be more resistant than usual to osmotic shock. The 400 mμ turbidity value of a phage preparation drops to 15 to 20 per cent of the original, following osmotic shock.

(*2*) *By Crenation.*—Anderson, Rappaport, and Muscatine (40) noted that the Tyndall scattering of a phage preparation disappeared when mixed with a strong sucrose solution. Contrary to the osmotic shock experiments, when strong sucrose has been added, dialysis or slow dilution does not leave phage in an infective form.

(*3*) *By Pyrophosphate.*—Dr. Helen Van Vunakis of this laboratory observed that dilute (10^{-4} to 10^{-2} M) pyrophosphate at pH 7 and 49°C. or pH 8.9 at 37°C. brought about a gradual drop in the infectivity and turbidity of aqueous or saline solutions of T2 phage at concentrations of 1 to 3 × 10^{11}/ml., and this was accompanied by a corresponding increase in viscosity of the solution, presumably from the liberated phage DNA. Such solutions were found to have ghosts that were indistinguishable from those produced by osmotic shock.

Precipitation of Ghosts.—Precipitation and settling of ghosts occur if following step 2 of Table I the solution is dialyzed at 5°C. in Visking membranes against 0.01 M acetate buffer of pH 4.4–0.05 M NaCl. The clear supernatant fluid can be easily separated from the precipitate and the latter sedimented at relatively low speeds. Resuspension of the residue and analysis of the lytic property have indicated a recovery of 85 per cent of the ghosts.

Microscopic Count.—Counts of bacteria were made in a Petroff-Hausser chamber using darkfield illumination and 4 mm. high dry objective lens. Occasionally a phase microscope was used.

Absorbancy Measurements.—These were the usual values obtained with the aid of a Beckman DU spectrophotometer standardized in the ultraviolet with 0.02 per cent A.C.S. reagent grade thiophene-free benzene in isooctane. The maxima were found at 249, 255, and 261 mμ which are within 2 mμ of the values in the International Critical Tables, volume 5, pp. 361–372.

Radioactive Measurements.—Samples of phosphorus and sulfur were dried at temperatures below 50°C. in metal planchets (Buckeye Stamping Co., Columbus, Ohio, Style No. 100) to which a few drops of alcohol were added to promote uniform spreading of the sample. These samples were then counted in a scaling instrument and after deducting the background count they were compared to a standard or ali-

quot of the starting material. The method had been previously shown to be free of the usual difficulties of self-absorption, spattering, etc. The instrument was standardized occasionally with a standard.

Preparation of Antisera.—

Antigens.—Purified phage (17) titering 2.7×10^{13}/ml. with a titer/$E_{400} = 2.4 \times 10^{12}$ was used in the preparation of ghosts.

"Shocked phage" consisted of shocking osmotically according to No. 1 in Table I followed by dialysis in a collodion membrane against water to remove the sodium sulfate. This was then concentrated through evaporation and addition of sodium chloride to 0.85 per cent. Most of the residual phage was removed by centrifugation at 9,000 × *g* for 1 hour. The phage titer of this preparation was 5×10^{7}/ml. and the ghost lytic titer was 5×10^{11}/ml.

Purified ghosts were prepared in accordance with the directions in Table I. Additional centrifugal fractionation was necessary to reduce the concentration of infective particles in the ghost preparation below 0.01 per cent of the ghost titer. Analyses of this preparation showed a ghost titer (lytic)/milliliter = 1.4×10^{13}; ghost titer/milligram N = 2.6×10^{13}; ghost titer/E_{280} (uncorrected) = 2.1×10^{12}; and phosphorus/N = 0.01, phage titer = 6×10^{8}/ml.

100 ml. quantities of the four antigens—(*a*) 3×10^{12}/ml. T2 phage; (*b*) 5×10^{11}/ml. shocked phage; (*c*) 1.4×10^{13}/ml. purified ghosts; (*d*) 6×10^{7}/ml. T2 phage control—were each mixed with 50 ml. of a suspension containing 10 ml. of 1 per cent $KAl(SO_4)_2 \cdot 10H_2O$, 0.6 ml./N/l KOH, 1.5 ml. 1 per cent merthiolate, and 37 ml. of saline. The final pH was 6.0.

Injection Schedule.—On the 1st and 2nd days 0.1 ml. and 0.5 ml. respectively of each antigen were injected intraperitoneally into three 10 pound healthy rabbits. After a 4 day lapse the animals were started with 0.5 ml. aliquots injected intravenously. Gradually increasing quantities were given by the same route 4 days a week for 4 weeks until they were finally receiving 5 ml. per injection. A total of 39 ml. was given to each animal. After a respite of 10 days the animals were exsanguinated from the heart and the serum collected after a clot formed at 5°C. in a large Petri dish.

SUMMARY

A method of preparing the protein coats or ghosts of phage T2 is described along with proof that the lytic action is a property of the ghost.

An assay based on the lytic action toward host cells has been developed which permits a rapid evaluation of the number of ghosts with a reliability of ±15 per cent.

The antigenic and certain physicochemical properties of the ghost have been determined.

The writers are grateful to their associates, Dr. I. R. Lehman and Dr. Helen Van Vunakis, for their helpful suggestions and for permitting us to report the results of some of their experiments.

To Patsy Miller Perlman and Mary T. Dunn we also owe a debt of gratitude for their patience and valuable technical assistance.

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ON THE MEASUREMENT OF CARBON DIOXIDE UPTAKE WITH A GLASS ELECTRODE

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(Received for publication, December 12, 1956)

A sensitive glass electode apparatus for measuring rapid changes in pH to determine the rate of carbon dioxide uptake by photosynthesizing cells was recently described (Rosenberg, 1954). An essential feature of this device was the elimination of a gas phase in equilibrium with the cell suspension and the consequent avoidance of the slow transport of carbon dioxide across the usually occurring gas-liquid interface. Results were reported on the influence of carbon dioxide on the rate of photosynthesis of *Chlorella pyrenoidosa*. These "transient method" experiments showed that the maximum rate of photosynthesis at high light requires local CO_2 concentrations corresponding to a gas with several tenths per cent CO_2 by volume. These results were confirmed by an independent steady state method involving the CO_2 analysis of a gas stream before and after it passed through a cell suspension of fixed pH.

Steemann Nielsen (1955) recently presented a summary of some of his experiments which indicated that the photosynthetic uptake of CO_2 by unicellular algae can reach its maximum rate at concentrations below 0.01 per cent CO_2. He ascribed the discrepancy between the findings of the two laboratories completely to systematic errors in my experiments. The purpose of the present article is to answer the specific criticisms and to suggest an alternative interpretation of the discrepancy.

1. The Transient Method

Steemann Nielsen objected to the use of dense suspensions of algae on the ground that cells at the rear of the vessel did not receive as much light as did cells at the front. For the particular purpose of showing carbon dioxide limitations, this objection has little validity. It is well known that carbon dioxide limitations of any origin become less important at decreasing light intensities. Any correction of the light shading would thus result in limitations even greater than those observed. The possibility of heating effects exaggerated by the use of dense suspensions, as suggested by Steemann Nielsen, was indeed eliminated in the original experiments by the use of infrared absorbing filters between the light source and the sample.

Steemann Nielsen feared that the short duration of an experiment in the

glass electrode compartment, about 2 minutes, might not be sufficient to allow for the dehydration of H_2CO_3 to CO_2, a known slow reaction. If so, the real concentration of CO_2 at the instant when a decreased rate of assimilation was observed might be appreciably below the equilibrium value assumed. This possibility was considered in the 1954 paper and was ruled out for all experiments carried out below pH 7.6. The rate constants for the dehydration reaction have been determined in almost a dozen laboratories in the past fifty years and are in remarkable agreement with each other. These data were the basis of the numerical calculations of the effect of slow dehydration reported in the 1954 paper in Table III. (The factor 2.3 should appear in the numerator of the right side of equation (7) of that paper. It was omitted through a typographical error but was correctly included in the computations for Table III.) Qualitatively, the reason for the unimportance of dehydration is as follows. At pH values below 7.9, almost all the variation of total inorganic carbon accompanying a variation in pH in a closed vessel is represented by a change in the equilibrium concentration of the species CO_2. The changes in HCO_3^- and $CO_3^=$ concentrations are negligible in comparison, and become important only above pH 8.0. During a 2 minute assimilation experiment in a closed vessel, as the pH rises inorganic carbon is removed from the medium. The equilibrium CO_2 concentration in the medium falls at a rate only slightly below the rate of fall of total inorganic carbon. The dehydration process must then operate at only a few per cent of the assimilation rate in order to maintain the equilibrium concentration of CO_2 in the medium. Thus there can be no appreciable difference between the true local concentration of CO_2 and the equilibrium concentration reported in Fig. 4.

2. The Steady State Method

The major criticism of the gas flow method was that CO_2 cannot diffuse fast enough from a gas bubble into the liquid medium so as to maintain the optimum rate of photosynthesis. If such were the case, the CO_2 concentration at the cell surface would be appreciably less than at the gas bubble, and a limitation in photosynthesis believed to occur at 0.2 per cent CO_2, for example, might really be associated with a true CO_2 concentration less than one-tenth this amount. This is certainly a valid question which requires a carefully considered reply.

Steemann Nielsen reported some experiments to show the slowness of diffusion from a gas bubble into the main body of a liquid. He bubbled normal air through a carbonate-bicarbonate buffer and measured the rate of uptake of CO_2 from the air stream. Only a fraction of the CO_2 in the air was absorbed, the amount depending on the pH of the medium. This type of experiment differs in two important respects from the steady state assimilation experiments. (1) In my assimilation experiments, the air was enriched so as to contain at least ten times as much CO_2 as normal air. (2) In a control air-buffer sys-

tem, CO_2 must be incorporated in the buffer largely by neutralization to HCO_3^- or $CO_3^=$. These hydration reactions are slow. In an air–buffer–algae system, on the other hand, CO_2 can be incorporated into the photosynthetic apparatus of the cells without necessarily going through the hydrated intermediate stage. Thus the removal of CO_2 from an air stream by a suspension of photosynthesizing cells will certainly be more efficient than by an inorganic alkaline buffer alone.

Mention was made in the 1954 paper of an attempt to calculate the actual extent of diffusion limitations in the steady state assimilation experiments. The calculation was made on the following premises. The diffusion barrier occurs at the liquid film surrounding each air bubble (Lewis and Whitman, 1924). The rate of diffusion of CO_2 across this film is proportional to the surface area of the bubble and to the difference in CO_2 concentration at the two sides of the film, with a proportionality constant k_L, called the specific film transfer coefficient. The concentration at the liquid side is C_L, the concentration in the main body of the liquid; at the bubble side it is C_g, the concentration in a hypothetical liquid equilibrated with the gas in the bubble. Equation (1) states the expected rate of CO_2 transfer from the air stream to the liquid on the above model.

$$J = (C_0 - C_L)\frac{v}{\beta}(1 - \epsilon^{-\alpha t_e}) \tag{1}$$

Here J is the rate of transfer, C_0 is the value of C_g at the air inlet where the air has its maximum CO_2 content, v is the volume rate of flow of the gas, β is the solubility of CO_2 expressed as the ratio of the equilibrium molar concentrations in liquid to gas, t_e is the time of rise of a single bubble through the suspension, and $\alpha = \frac{k_L A \beta}{V}$, in which A is the area and V the volume of a single bubble. In my paper I estimated an average value of 1.4 for (αt_e). This was the basis of my statement on the extent of the diffusion limitations in my original measurement.

Subsequently Dr. Warren Butler (private communication), working in the laboratory of Dr. Franck and Dr. Gaffron, directly measured the diffusional rate for a geometrical arrangement identical with the one used in my original work and found an experimental value for (αt_e) of 0.94. He measured the fractional saturation of an air stream with CO_2 when bubbled through a carbonate-bicarbonate buffer. The figure 0.94 is a minimum value because it did not include a correction for the slowness of the CO_2 hydration and dehydration reactions in inorganic systems. The correction would raise the value of (αt_e) to somewhere between 1.0 and 1.2. If the lower value in this range is accepted, there would be a downward revision of the values for the CO_2 content listed in Figs. 6 and 7 of the 1954 paper, each point being lowered by about 0.15 per cent CO_2, but limitations would still be observed at several

tenths per cent CO_2. The adjusted data for photosynthesis in 0.002 M bicarbonate are plotted in Fig. 1.

If Steemann Nielsen's description of these experiments were correct, that the CO_2 in the medium was depleted below 0.01 per cent, the rate of absorption of CO_2 from the gas bubbles should have been within 2 per cent of the maxi-

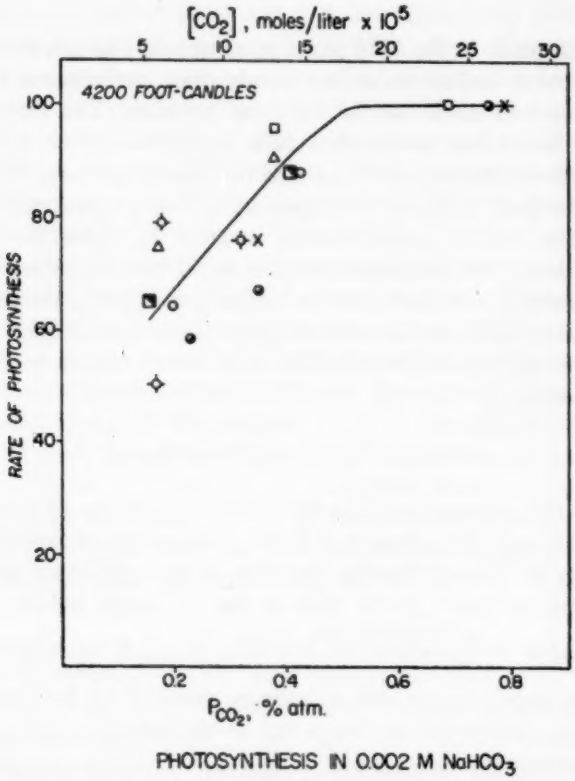

FIG. 1. Photosynthesis of *Chlorella* in 0.002 M $NaHCO_3$. Average density of cells, 10 µl. per ml. Each symbol represents a particular harvest of algae on a single day. All points of the same symbol correspond to a set of measurements with the same preparation.

mum for a similar gas stream through a medium completely devoid of CO_2. In terms of equation (1) this maximum fractional absorption is $(1 - e^{-at_s})$. In the experiments showing the greatest limitations in Fig. 1, the actual utilization of CO_2 was 40 per cent, whereas the maximum possible absorption from this type of gas stream has been shown to be over 63 per cent. In other words, depletion of the medium was never enough to lower the actual CO_2 concentration in solution to values as low as 0.1 per cent. The estimate in the 1954 paper of the importance of diffusion limitations was not sufficiently

cautious, but the resulting error in the CO_2 concentration was not nearly as large as suggested by Steemann Nielsen. For absolute measurements there is no doubt that the transient method is more reliable in the region of its applicability, but the steady state method is sufficiently reliable to confirm the surprising result that limitations can occur up to several tenths per cent CO_2.

3. Interpretation

It would seem more reasonable to ascribe the discrepancy between the two laboratories to a biological difference than to an experimental one. Whittingham (1952) and Briggs and Whittingham (1952) pointed out that long adaptation periods, of the order of an hour, are often required for algae to make efficient use of carbon dioxide at low pressures. Gaffron (private communication) found that adaptation over similar periods is required even at carbon dioxide pressures up to 0.5 per cent atmosphere in algae that had been pretreated in the dark with 50 per cent carbon dioxide. Brackett and Olson (private communication) have consistently observed limitations in ordinary air in the photosynthetic rates of *Chlorella* grown in a 5 per cent carbon dioxide atmosphere, if the long adaptation had not been allowed to proceed. It should not be surprising, therefore, that algae grown in ordinary air by Steemann Nielsen may have much more facile response to low carbon dioxide than similar algae grown in 4 per cent carbon dioxide like those used in my original work. Also, his experiments lasted up to several hours. My experiments lasted only a few minutes in the transient method and about 20 minutes in the steady state method.

SUMMARY

1. It has been shown that the glass electrode method as previously described by the author for measurements of transient rates in photosynthesis is free of systematic errors.

2. Justification has been given for the use of the steady state–gas flow method for measuring rates of assimilation under conditions in which the solution is known not to be seriously depleted of CO_2.

3. The decline of photosynthetic rates under light saturation at CO_2 pressures less than several tenths per cent of an atmosphere has been shown to be a real phenomenon. It has been suggested that there may be a real discrepancy between this finding and those of some other investigators due to a difference in the previous history of the algal suspensions.

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THE CHEMISTRY OF INSECT HEMOLYMPH

II. Trehalose and Other Carbohydrates*

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(Received for publication, February 11, 1957)

A curious feature of insect blood, shown by many analyses, is the apparent absence of all but minute amounts of sugar (for reviews, see Beutler, 1939; Babers, 1941; Buck, 1953). Reducing substances are abundant, but (except in the case of a few species) the greater part of these are not fermentable by yeast and are therefore presumably not sugar. Typical are data on the silkworm, *Bombyx mori*, in which the reported levels of blood sugar range from zero up to about 30 mg. per 100 ml. (Florkin, 1937; Kuwana, 1937; and others) —remarkably small amounts for a metabolically active animal with a high dietary intake of sugars. Twenty years ago, however, Kuwana (1937) made the significant discovery that acid hydrolysis of *Bombyx* hemolymph caused release of much reducing sugar from a substance which was not glycogen. Even earlier, Ronzoni and Bishop (1929) had reported an unidentified non-reducing "polysaccharide below glycogen" in the blood of honeybee larvae. These reports received little attention. Recently, unaware of them, we made essentially the same finding. The blood of *Bombyx mori* and of two other insect species was found to contain large amounts of anthrone-reactive material which was neither a reducing sugar, sucrose, nor glycogen (Wyatt, Loughheed, and Wyatt, 1956).

As already reported in preliminary form (Wyatt and Kalf, 1956), we have now identified as a major sugar of insect plasma the non-reducing disaccharide α,α-trehalose. In the present paper, we give details of the isolation and characterization of this sugar together with some quantitative analyses of trehalose and certain other carbohydrates in insect plasma. Their distribution and significance will be considered.

* This work was supported by a research grant from the United States Public Health Service and by a grant from the Medical Fluid Research Fund of the Yale University School of Medicine. Some of the later experiments were performed and the results were prepared for publication during the tenure by one of us (G.R.W.) of a John Simon Guggenheim Memorial Foundation fellowship at the Biological Laboratories, Harvard University.

‡ United States Public Health Service Predoctoral Research Fellow of the National Cancer Institute.

Materials

Larvae of the silk moths, *Telea polyphemus* and *Platysamia cecropia*, were reared in outdoor cages on maple and wild cherry respectively; cocoons containing diapausing pupae were obtained from dealers. Larvae of *Drosophila repleta* were provided by Professor D. F. Poulson. Specimens of the milkweed bug, *Oncopeltus fasciatus*, the flour beetle, *Tribolium confusum*, the mosquito, *Aedes aegypti*, and the tobacco worm, *Protoparce sexta*, were obtained through the courtesy of Mr. Neely Turner and other

TABLE I

Properties of Hemolymph Plasma Samples

Sample No.*	No. of insects	Sex	Stage	Dry matter
				gm./100 ml.
Tp 11	15	Mixed	Mature larvae	7.6
Tp 13a	3	♂	Pupae, kept 7 mos. at 5°C.	12.4
Pc 15	66	Mixed	Mature larvae	7.9
Pc 7a	3	♂	Pupae kept 5 mos. at 5°C.	16.0
Pc 7b	3	♀	Pupae kept 5 mos. at 5°C.	15.7
Pc 10a	3	♂	Pupae kept 4 mos. at 5°C., then 5 days at 25°C.	12.7
Pc 10b	3	♀	Pupae kept 4 mos. at 5°C., then 5 days at 25°C.	17.0
Pc 21a	6	♀	Pupae, from the same collection as Pc 7 and Pc 10, kept 12 mos. at 5°C.	15.5
Pc 21b	6	♀	Developing adults (after 12 mos. diapause), 10th day‡	12.6
Pc 21c	4	♀	Developing adults (as above), 15th day	17.0
Pc 21d	6	♀	Developing adults (as above), 20th day	12.6

* Tp, *Telea polyphemus*; Pc, *Platysamia cecropia*.

‡ Stages in adult development according to the time-table of Schneiderman and Williams (1954).

members of the staff of the Connecticut Agricultural Experiment Station, New Haven.

Lyophilized plasma was prepared as previously described (Wyatt, Loughheed, and Wyatt, 1956). Samples used for quantitative analysis are listed in Table I. Analyses were also carried out on some of the samples described in the earlier paper (Bm 7, 11, 12, and 14, are from larvae of the oriental silkworm, *Bombyx mori*; Bm 15, from pupae of *B. mori*; Gm 1, from larvae of the wax moth, *Galleria mellonella*; Dh 1, from larvae of the spruce sawfly, *Diprion hercyniae*).

Methods

Preparation of Deproteinized and Deionized Extracts

Large samples of hemolymph for isolation of trehalose were deproteinized by adding an equal volume of 0.6 N $HClO_4$ and centrifuging. The precipitates were reextracted with 0.3 N $HClO_4$, then the combined extracts were neutralized with KOH, chilled,

and freed of $KClO_4$ on the centrifuge. Solutions were then deionized by passage first through a bed of Dowex 1 (× 10, formate) and then a bed of Dowex 50 (× 12, acid). Neutral carbohydrates were washed from the columns with water, and their distribution in the effluents was followed by analysis with anthrone.

Smaller volumes of insect blood or of tissue extracts for paper chromatography and electrophoresis were usually freed of protein and glycogen with hot ethanol (final concentration 60 to 70 per cent) and then deionized with a small column containing a layer of each of the above resins. Effluent solutions were dried in a stream of air; the residues were rinsed with petroleum ether to remove lipides and then redissolved in small volumes of water. In a few cases, protein was coagulated in aqueous extracts of tissues by heating at 100° C.; such solutions, however, tended to clog the resin bed.

Extracts for quantitative chromatography were prepared from about 10 mg. of lyophilized plasma. This was extracted 3 times with 0.4 ml. portions of 70 per cent ethanol, the mixture being heated each time to 75° C. for 5 minutes. The extracts were applied successively to a layered deionizing column (4 mm. × 50 mm. containing Dowex 1 and Dowex 50) and elution was completed with a further 0.4 ml. of 70 per cent ethanol. After evaporation of the effluent solution, the residue was carefully rinsed with 0.5 ml. of petroleum ether and redissolved in 0.05 ml. of water. 10 μl. portions were used for chromatography.

To prepare samples for quantitative determination of trehalose by chemical isolation, 5 to 10 mg. of lyophilized plasma was suspended in a measured volume (usually 1 to 2 ml.) of 60 per cent ethanol. The tube was stoppered, heated at 75° C. for 5 to 20 minutes, and then centrifuged. Portions of 0.1 ml. were pipetted into pyrex test tubes and evaporated to dryness before analysis. Removal of ethanol was necessary since its presence was found to enhance the color from sugars in the anthrone reaction. In a few experiments, portions of such extracts were deionized by passing them through layered resin columns (as above), the effluent being evaporated and redissolved in water before taking samples for analysis.

Paper Chromatography

Whatman No. 1 paper was used, generally in the descending technique, the solvent being allowed to drip from the serrated lower end of the paper. A solvent system which we found satisfactory for qualitative and quantitative work is described in Table II. Good distribution of R_G values, but slower mobility, was given by *n*-butanol–acetic acid (Partridge, 1948). We also occasionally used ethyl acetate–pyridine–water (Jermyn and Isherwood, 1949) and butanol–ethanol–acetone–water (Gray and Fraenkel, 1954), in the ascending technique, but these fast-flowing systems gave, in our experience, rather diffuse spots.

For detection of carbohydrates including trehalose and glycogen the permanganate-periodate reagent of Lemieux and Bauer (1954) was used. To prevent migration of the spots it was found advantageous to spray the papers while they rested horizontally on a rack. For detection of sugars other than trehalose, benzidine trichloroacetate (Bacon and Edelman, 1951) was found useful. After chromatography, 1 μg. of maltose was detectable by this reagent, whereas as much as 300 μg. of trehalose gave no visible spot.

In the earlier stages of the study, trehalose and glucose were determined by quanti-

tative chromatography. For this purpose, the paper was marked in lanes 3 cm. wide. Each sample to be analyzed was spotted on each of three lanes, one of which was next to the margin of the paper. Two lanes were always left blank. After running the chromatogram, the marginal lanes were cut off, treated with periodate-permanganate reagent, and then used to locate the sugars in the unsprayed lanes. The appropriate areas were cut out, usually as 3 cm. squares, and the sugars eluted with about 0.5 ml. of water (Consden, Gordon, and Martin, 1947). The eluates were evaporated to dryness in a desiccator and analyzed with anthrone. Blanks from 9 sq. cm. of paper gave color equivalent to 4 to 6 μg. of glucose; this value was not materially lowered by prior washing of the paper. The procedure was tested with a solution containing

TABLE II

Chromatographic Separation of Sugars

Solvent system: 80 per cent (by volume) *sec.*-butanol in water, run at 37°C. for about 44 hours. Movement of glucose: 10 to 14 cm. R_G = movement of sugar/movement of glucose.

Sugar	R_G	Sugar	R_G
Raffinose	0.13	Galactose	0.90
Melezitose	0.20	Glucose	1.00
Trehalose	0.38	Mannose	1.24
Maltose	0.39	Fructose	1.28
Cellobiose	0.39	Arabinose	1.35
Sucrose	0.56	Ribose	1.91
		N-Acetylglucosamine	1.56

2.68 mg. glucose and 15.41 mg. trehalose per ml. Four analyses gave: glucose, mean 2.54, range 2.25 to 2.73 mg. per ml.; trehalose, mean, 16.0, range 15.1 to 17.4 mg. per ml.

Electrophoretic Separation of Sugars

Since paper chromatography failed to resolve trehalose from maltose and cellobiose, we used electrophoretic mobility in borate (Consden and Stanier, 1952) as a further means of separating sugars. Solutions were applied to a strip of Whatman No. 1 paper, which was moistened with 0.02 M $Na_2B_4O_7$ and then immersed in CCl_4 in the apparatus of Markham and Smith (1952). A potential gradient of 40 v. per cm. was applied for 2 hours. The paper was dried, sprayed with dilute ethanolic acetic acid to neutralize the borate, and again dried before spraying with permanganate-periodate reagent. Trehalose and sucrose, which would not be expected to form borate complexes, moved about 4 cm. toward the cathode, presumably because of migration of the buffer. Relative to the position of trehalose, cellobiose moved 3 cm., maltose 5.5 cm., and glucose 29 cm. toward the anode.

Chemical Isolation of Trehalose for Quantitative Determination

In later quantitative analyses, we supplanted the rather tedious chromatographic isolation of trehalose by a procedure which takes advantage of the exceptional sta-

bility of this sugar to both acid and alkali. Samples containing 20 to 75 μg. of trehalose were evaporated to dryness in pyrex tubes and redissolved in 0.2 ml. of 0.1 N H_2SO_4. Each tube was capped with aluminum foil and heated at 100° C. for 10 minutes to hydrolyze any sucrose or glucose-1-phosphate. The solution was made alkaline by addition of 0.15 ml. of 6 N NaOH and again heated at 100° C. for 10 minutes to destroy reducing sugars. The sample was then chilled, 2.0 ml. of anthrone reagent was added, and the analysis completed as described below. Trehalose standards and blank tubes were carried through the acid and alkaline treatments. Loss of trehalose during this treatment is small (about 5 per cent). A number of carbohydrates were tested for interference in amounts of 100 μg. and 500 μg. Color yields per 100 μg. of carbohydrate, expressed as micrograms of trehalose giving equal color, were: glucose, galactose, sorbose, lactose, sucrose, melezitose, glucosamine, *N*-acetylglucosamine, glucose-1-phosphate, glucose-6-phosphate, arabinose, ribose, rhamnose, less than 1; mannose, fructose, maltose, cellobiose, fructose-1,6-diphosphate, 1 to 2; raffinose, 5. Glycerol, which gives an orange color with the anthrone reagent (Schutz, 1938), does not interfere at 630 mμ.

Determination of Glycogen

The alcohol-insoluble residues after extraction of sugars from plasma were suspended in 0.1 ml. of 30 per cent KOH and heated at 100° C. for 20 minutes. 0.4 ml. of 80 per cent ethanol was added, and the mixture was warmed to flocculate the precipitate, and centrifuged. The precipitate was washed once with absolute ethanol, dried, and its glycogen content determined with anthrone. It is possible that the fraction thus isolated may contain anthrone-reactive substances other than true glycogen (Balmain, Biggers, and Claringbold, 1956).

Anthrone Reaction

Anthrone reagent was prepared according to Mokrasch (1954) and used by mixing 2.0 ml. of reagent with 0.35 ml. of aqueous sample in a 13 mm. pyrex tube. For use with dried samples, the reagent was diluted with one-sixth its volume of water. Aldose and ketose could be distinguished by reading the color after different periods of heating (Mokrasch, 1954); when this was not necessary, the tubes were heated at 100° C. for 15 minutes, then cooled to room temperature. With solutions which had contained NaOH, excessive chilling led to crystallization of sodium sulfate. Color was read in the Beckman spectrophotometer at 630 mμ or in the Klett-Summerson colorimeter (adapted for 13 mm. tubes) with No. 66 filter.

Isolation and Determination of Glucose-6-Phosphate

In order to account as completely as possible for the carbohydrate content of some samples of insect blood, and to confirm the earlier inference that larval *Bombyx* blood contains glucose-6-phosphate (Wyatt, Loughheed, and Wyatt, 1956) we performed some analyses by ion exhcange chromatography. Plasma was deproteinized with $HClO_4$ and the extract was applied to a column of Dowex 1 (× 10, formate) and eluted with a concentration gradient of formic acid (Hurlbert, Schmitz, Brumm, and Potter, 1954). Fractions were collected and analyzed for carbohydrate with anthrone

and for phosphorus (method of Lowry, Roberts, Leiner, Wu, and Farr, 1954, scaled to 2 ml. volume).

Glucose-6-phosphate was determined enzymically in fractions from the columns and in unfractionated $HClO_4$ extracts of insect plasma by spectrophotometric measurement of reduction of triphosphopyridine nucleotide coupled with the action of glucose-6-phosphate dehydrogenase. When results were negative, the absence of inhibitory substances was demonstrated by addition of known glucose-6-phosphate. Enzyme and coenzyme were obtained from the Sigma Chemical Co., St. Louis, Missouri, and the reaction was carried out as recommended in Sigma Bulletin No. 201.

RESULTS

Isolation and Characterization of Trehalose from Hemolymph

From 100 diapausing pupae of *Telea polyphemus*, 63.4 gm. of plasma was obtained. After removing protein and ionic substances, the solution was lyophilized, yielding 958 mg. of crude carbohydrate. The chief anthrone-reactive substance was shown to be freely dialyzable, and therefore not glycogen. It did not reduce Benedict's reagent. After chromatography on paper it could not be detected by spraying with benzidine trichloroacetate or with aniline phthalate (Partridge, 1949), but with permanganate-periodate reagent a spot appeared having a mobility similar to that of maltose. After hydrolysis (N H_2SO_4, 105°C., 1½ hours) glucose was the sole product detected by chromatography. These properties suggested that the substance might be trehalose.

For purification, 400 mg. of the crude carbohydrate was dissolved in 3.0 ml. of water. Since analysis of the effluent from the columns had shown incomplete separation of carbohydrates from phosphorus-containing compounds, the latter were precipitated by addition of 1.6 ml. of saturated $Ba(OH)_2$ followed by ethanol to a concentration of 70 per cent and heating. A flocculent precipitate was centrifuged off, and the excess of barium was removed as the carbonate. The alcoholic solution was stored at 3°C. After 24 hours a small amorphous precipitate was removed and the solution was evaporated to dryness. The residue was dissolved in 0.5 ml. of water and absolute ethanol was slowly added. At 66 per cent concentration, a fine amorphous precipitate formed and was removed on the centrifuge. Alcohol was added to a concentration of 80 per cent and the clear solution was left in the cold. After 24 hours, large colorless rhombic crystals had formed. These were collected, recrystallized twice from 80 per cent ethanol, and dried; yield, 121 mg. *Anal.* Loss on drying at 105°, 9.07. Calcd. for $C_{12}H_{22}O_{11} \cdot 2H_2O$: H_2O, 9.52. The anhydrous material gave C, 41.44; H, 6.40. Calcd. for $C_{12}H_{22}O_{11}$: C, 42.10; H, 6.48. M.P. 96.8–97.2, corr. Optical rotation (for the dihydrate) $[\alpha]_{22}^{D}$ + 184° (H_2O, *c* 1). Commercial trehalose dihydrate, twice recrystallized, gave M.P. 96.5–97.5, $[\alpha]_{22}^{D}$ + 174°. The sugar isolated from hemolymph migrated identically with commercial trehalose when subjected to paper chromatography in four solvent systems.

Trehalose Octaacetate.—This derivative was prepared from 20 mg. of the isolated trehalose with acetic anhydride in pyridine, by an adaptation of the procedure of Pangborn and Anderson (1933). The product was twice recrystallized from ethanol, yielding 11 mg. of colorless needles, M.P. 78–79°. An acetate prepared by the same procedure from commercial trehalose had identical

TABLE III

Presence of Trehalose in Various Organisms

Organism	Material examined	Trehalose found	Remarks
Insects			
Oncopeltus fasciatus (Hemiptera)	Whole adults	+	
Tribolium confusum (Coleoptera)	Whole adults	+	
Aedes aegypti (Diptera)	Whole larvae	+	
Drosophila repleta (Diptera)	Whole larvae	+	
Diprion hercyniae (Hymenoptera)	Larval plasma	+	
Galleria mellonella (Lepidoptera)	Larval plasma	+	
Bombyx mori (Lepidoptera)	Larval and pupal plasma	+	
Protoparce sexta (Lepidoptera)	Larval plasma	+	
Telea polyphemus (Lepidoptera)	Larval and pupal plasma	+	
Platysamia cecropia (Lepidoptera)	Pupal plasma	+	
Other			
Lobster (*Homarus americanus*)	Plasma	−	Limit of detectability: 10 mg. per 100 ml.
Human	Plasma	−	Limit of detectability: 5 mg. per 100 ml.
Tobacco	Fresh leaves	−	

crystal form and melting point, which correspond to those of trehalose octaacetate (Pangborn and Anderson, 1936).

Recognition of Trehalose in Other Species of Insects

Recognition of trehalose in other species rests on the following criteria: (1) by paper chromatography of a deproteinized, deionized extract, a substance was demonstrated having the mobility of trehalose and reacting with the periodate-permanganate reagent, but not reacting with benzidine trichloroacetate; (2) the substance eluted from a paper chromatogram and examined by electrophoresis in borate exhibited the immobility characteristic of trehalose; (3) the substance when eluted from a paper chromatogram, hydrolyzed with

H_2SO_4, and rechromatographed, gave glucose as the sole product. As shown in Table III, trehalose was found in all the insects examined, but not in preparations from a crustacean, a mammal, nor a flowering plant.

Characterization of Glucose-6-Phosphate from Hemolymph

Anion exchange fractionation has been applied as yet only to plasma from *T. polyphemus* pupae and from *Bombyx mori* larvae. With the former, all of the carbohydrate passed through the columns with the effluent front, indicating the absence of phosphorylated sugars. In the *Bombyx* extracts, however, a peak containing both carbohydrate and phosphorus came off the columns at the position in the acid gradient expected of a hexose monophosphate. This

TABLE IV

Analysis of Fraction from Bombyx Hemolymph Containing Glucose-6-Phosphate

	Total P	10 min. labile P*	Aldohexose (anthrone)	Ketohexose (anthrone)	Glucose-6-phosphate (enzymic)
	μmol. per ml. solution				
Fraction from hemolymph	13	0	7.6	2.2	6.2
Known glucose-6-phosphate	7	0	6.8	0.1	6.1

* 1 N H_2SO_4, 100°C.

fraction, after concentration, gave the analyses shown in Table IV. These indicate the presence of glucose-6-phosphate together with other stable phosphate compounds, apparently including a ketose derivative. Paper chromatography (Wyatt, Loughheed, and Wyatt, 1956) also showed a major component migrating identically with glucose-6-phosphate. Because of lack of material, further fractionation was not attempted.

Quantitative Content of Carbohydrates in Hemolymph

The results of various quantitative determinations are summarized in Table V.

The values for total sugars were obtained by anthrone analysis, before chromatographic or chemical isolation of trehalose, on samples of the substances extracted from lyophilized plasma by 60 to 70 per cent ethanol. For the chromatographic method, these solutions had been deionized; for the chemical method, they were deionized in only a few cases, as indicated. The close agreement between total carbohydrate and trehalose content in the extracts from *Bombyx* plasma (even without deionization) must signify that glucose-6-phosphate was not extracted from the protein.

It will be noted that when both methods of trehalose analysis were used on the same sample, results from the chemical method are higher by 12 to 40

per cent. That the two methods give similar ratios of trehalose to total sugars (in all samples except Dh-1) suggests that both afford satisfactory isolation of trehalose, and that the differences arose in preparation of the extracts. One difference in procedure is that the dried extracts for chromatography were

TABLE V

Quantitative Analyses of Carbohydrates of Hemolymph

0, none detectable; —, not tested. Except when indicated, values are averages of two or more replicate analyses.

Sample No.	Chromatographic method			Chemical method		Glycogen	Glucose-6-phosphate‡
	Total sugars*	Trehalose	Glucose	Total sugars	Trehalose		
	Mg./100 ml. plasma						
Bm 7a	—	—	—	305	306	—	88
Bm 7b	—	—	—	397	407	—	121
Bm 12	427	419	3	530	523	12	39
Bm 14a	—	—	—	453	419	—	—
Bm 11	451	442	2	—	—	15	—
Bm 15	200	202	0	—	—	38	—
Gm 1	1356	1312	21	1766	1703	41	0
Dh 1	894‡	664‡	28‡	1089	926	32‡	0
Tp 11	—	—	—	1100, 1017*	1036	—	0
Tp 13a	—	—	—	1408, 1304*	1398, 1324*	—	0
Pc 15	—	—	—	1255	1194	—	—
Pc 7a	439	406	—	587	581	25	14
Pc 7b	536‡	530‡	—	619, 559*	596, 561*	31‡	—
Pc 10a	502‡	475‡	0	620	539	31‡	0
Pc 10b	436‡	416‡	8	598	545	55‡	0
Pc 21a	—	—	—	987	949	—	—
Pc 21b	—	—	—	709	652	—	—
Pc 21c	—	—	—	1046	966	—	—
Pc 21d	—	—	—	1199	1159	—	—

* Deionized.

‡ Single analysis only.

washed with petroleum ether; although sugars are insoluble, some fragments of the residue may have been dislodged and lost. Another factor may be deionization, for deionization of a few extracts before analysis by the chemical method lowered the results by 5 to 10 per cent, suggesting removal of interfering substances, perhaps organic acids. Sugar standards passed through similar columns were recovered quantitatively. It seems likely that the true trehalose levels may be intermediate between the values obtained by the two procedures.

It is also apparent that the present results are lower than those reported

earlier for total carbohydrate in some of the same hemolymph samples (Wyatt, Loughheed, and Wyatt, 1956). Three sources of difference are evident. The earlier extracts were made with acid instead of alcohol and thus included glycogen and glucose-6-phosphate. We have also subsequently found that the trichloroacetic–perchloric acid mixture interferes slightly in the anthrone reaction, enhancing color yield by about 5 per cent. A further difference (5 per cent, for water eliminated in the glycosidic link) is due to the earlier data being expressed as glucose rather than trehalose.

In all the blood samples analyzed from lepidopterous insects (*B. mori*, *G. mellonella*, *T. polyphemus*, and *P. cecropia*) trehalose accounted for more than 90 per cent of the total blood sugar (except Pc 10a by the chemical method, for which the value is 87 per cent). In several samples, the fraction represents 100 per cent, within experimental error. In the sawfly *Diprion hercyniae* the proportion of blood sugar represented by trehalose is less (74 per cent by the chromatographic method, 85 per cent by the chemical method). Chromatograms from this species showed two unidentified periodate-reactive spots, one migrating more slowly than trehalose (possibly a trisaccharide) and one moving rather faster than glucose; it is possible that these substances include a carbohydrate which resists acid and alkaline degradation.

DISCUSSION

Trehalose is know to occur in many fungi and is commonly regarded as a product characteristic of lower plants (Myrbäck, 1949). Nearly one hundred years ago, this sugar was isolated from cocoons (collected in Syria and known as "*trehala*") of the weevil *Larinus nidificans* (Berthelot, 1859), and more recently it has been identified in another "desert manna" believed to be the excretion of a scale insect (Leibowitz, 1944). Nevertheless, the possibility of its occurring in insect blood and having a normal role in insect metabolism has apparently not been considered before the present investigation. It is now clear that trehalose is widely distributed among insects. Since our preliminary publication, trehalose has been reported in the locust, *Schistocerca gregaria* Howden and Kilby, 1956), the blowfly *Phormia regina*, and the honeybee (Evans and Dethier, 1957). In the species that we examined quantitatively, it is the major blood sugar. This is not true of all insects, however, for the principal blood sugar of *Gastrophilus* is fructose (Levenbook, 1950) and the blood of adult bees contains very large amounts of glucose together with some fructose (Beutler, 1936; von Czarnowski, 1954).

Trehalose has also recently been found in invertebrates other than insects. Fairbairn and Passey (1957) have isolated crystalline trehalose from *Ascaris* and have demonstrated this sugar chromatographically in several other parasitic worms. On the other hand, we were unable to detect trehalose in the blood of a lobster.

Of the insect species which we examined, only one (*Drosophila*, feeding on yeast) is known to have a source of trehalose in its diet. We have been unable to detect trehalose in tobacco leaves, the food of the tobacco hornworm. Insects must therefore synthesize this sugar. Synthesis of trehalose from glucose undoubtedly accounts for the observations of Kuwana (1937), who fed glucose to silkworms and followed the subsequent changes in blood sugar. Reducing sugar reached a maximum 4 hours after feeding and then fell sharply, but the fall in reducing sugar was accompanied by a rise in the level of a substance which gave reducing sugar upon hydrolysis: this reached a maximum some 10 hours after feeding, and was still elevated after 24 hours. The rapid conversion of other sugars to trehalose is also signalled by the fact that *Galleria* larvae, on a diet containing honey, have 1.5 per cent of trehalose in their blood and only 0.02 per cent of glucose.

That various insects are capable of utilizing trehalose is known. Bee larvae and adults can metabolize ingested trehalose efficiently, as indicated by measurements of survival time (Bertholf, 1927; Phillips, 1927; Vogel, 1931). It will also maintain adult blowflies (Fraenkel, 1940; Hassett, Dethier, and Gans, 1950) and *Drosophila* (Hassett, 1948); in *Drosophila* it is one of the few sugars which will support continuous flight (Wigglesworth, 1949). Moreover, aphids contain an enzyme which digests trehalose (Duspiva, 1954). These results could be attributed to the presence of non-specific digestive α-glucosidases in insects. However, trehalose is at least as good a substrate as glucose for respiration of housefly flight muscle homogenates (Sacktor, 1955), and Frèrejacque (1941) found that enzyme preparations from a number of insect species split trehalose much more rapidly than sucrose or maltose, and that the rate is increased by the presence of phosphate. This last finding, which we have confirmed, might be explained by phosphorolytic cleavage; such a mechanism would be of evident physiological value, permitting conservation of the energy of the glycosidic linkage. A study of the metabolism of trehalose by insect tissues will be reported in a later communication.

It is of interest to compute what fraction of an insect's carbohydrate reserve is in the form of trehalose, and how this changes during metamorphosis. If one assumes (1) that the total carbohydrate in the mature larva of the Cecropia silkworm consists of tissue glycogen and blood trehalose, (2) that glycogen comprises 1 per cent of the fresh weight, as in *Prodenia* (Babers, 1941), and (3) that the blood, making up 30 per cent of the body weight, contains 1 per cent of trehalose, then trehalose would account for some 23 per cent of the total carbohydrate of the insect. In the pupa, if glycogen has risen to 2 per cent of the body weight, as in *Prodenia* and in *Antheraea pernyi* (Drilhon, 1935), and the blood contains 0.5 to 1.0 per cent of trehalose, then the fraction of total carbohydrate accounted for by trehalose becomes 7 to 13 per cent. These approximations are probably representative of the changes occurring at the

time of pupation in many lepidopterous insects. They suggest conversion of trehalose into glycogen. This possibility is significant in relation to a long-standing problem; namely, the source of the net increase in glycogen that occurs in various insects after the cessation of larval feeding. Increase in glycogen at this time was noted, for example, in *Bombyx* (Bataillon and Couvreur, 1892), in *Prodenia* (Babers, 1941), and in *Popillia* (Ludwig and Rothstein, 1949). An interpretation which originates with Couvreur (1895) and recurs in the entomological literature postulates that glycogen is synthesized from fat. Whether net conversion of fat to carbohydrate does in fact take place in insects is of great interest. Mammals are apparently incapable of this process (Deuel and Morehouse, 1946), but it does occur in plants (Murlin, 1933; Christiansen and Thimann, 1950), and evidence has recently been adduced in support of its occurrence in *Ascaris* (Passey and Fairbairn, 1956). In the analyses which suggest the conversion of fat to glycogen in insects, trehalose was overlooked. A decision as to whether utilization of trehalose can account quantitatively for the glycogen synthesis during pupation must await a thorough analytical study.

In the Cecropia pupa, our analyses (Pc 7a and 7b, 10a and 10b) fail to show any significant difference between the levels of trehalose in blood of male and female diapausing animals. An increase in blood trehalose during prolonged storage at 5°C., presumably reflecting conversion from glycogen, is indicated by the analysis of sample Pc 21a. Samples Pc 21b to 21d, taken at intervals during adult development, show a fall, then a rise, in blood trehalose concentration. To obtain total blood sugar one must, of course, take into account the blood volume, which decreases during adult development. The change in blood volume has not been accurately measured in Cecropia, but it is indicative that the average yield from bleeding fell progressively from 28 per cent of body weight in diapausing pupae to 10 per cent on the 20th day of development. Multiplying the sugar concentrations by these values indicates that the total trehalose may drop by adult emergence to about half of its amount in the pupa. The proportional decline in trehalose during adult development is thus less than that of glycogen, which falls by the time of emergence to one-fourth or less of its level in the pupae (*Bombyx*, Needham, 1929; *Antheraea pernyi*, Drilhon, 1935). Evidently, trehalose plays a relatively minor role as an energy reserve during metamorphosis. It may be more significant in carbohydrate transport, perhaps conveying glucose units from fat body glycogen to sites of metabolism in other tissues.

Since various mycobacteria contain lipides which are esters of fatty acids with trehalose (see Myrbäck, 1949), the question arose whether such lipids might be found also in insects. In unpublished experiments by G. R. Wyatt and W. L. Meyer, lipides were extracted from *T. polyphemus* pupae with ethanol ether and saponified. Chromatography of the products revealed large amounts of glycerol but failed to detect trehalose.

Whereas trehalose accounts for the greatest part of the carbohydrate in the plasma samples which we examined, several other carbohydrates are present. Glucose occurs in small amounts. The trace amounts of fructose and sucrose previously reported in *Bombyx* (Wyatt, Loughheed, and Wyatt, 1956) are below the limits detectable by our present methods. Apparent glycogen occurs in small amount in larval blood, increasing after pupation (Bm 15) presumably owing to histolysis. Glucose-6-phosphate, the presence of which in insect plasma has previously been indicated (Smolin, 1952; Wyatt, Loughheed, and Wyatt, 1956), has now been characterized by a specific enzymic method, and evidence has been obtained for smaller amounts of a ketose phosphate. In view of Faulkner's (1956) demonstration of phosphohexose isomerase in *Bombyx* blood, the latter is likely to be fructose-6-phosphate. In larval *Bombyx* hemolymph, glucose-6-phosphate can occur at quite high levels; it undoubtedly serves as a substrate for the triphosphopyridine nucleotide-linked sugar phosphate reductase of *Bombyx* plasma recently described by Faulkner (1956).

SUMMARY

α,α-Trehalose, a sugar previously regarded as a product characteristic of certain lower plants, has been identified as a major blood sugar of insects. Trehalose has been isolated in pure form from the blood of pupae of the silk moth, *Telea polyphemus*, and has been recognized chromatographically in all the insects examined, which comprise 10 species belonging to 5 different orders. Trehalose has been determined quantitatively with anthrone after either chromatographic separation or chemical degradation of other sugars. In larvae and pupae of 4 species of Lepidoptera it ranges from 0.2 to 1.5 gm. per 100 ml. of blood and makes up over 90 per cent of the blood sugar; in larvae of a sawfly, about 80 per cent of the blood sugar is trehalose. In *Bombyx mori* and *Platysamia cecropia*, the pupal blood trehalose level is about half that in the mature larva, suggesting utilization of trehalose for glycogen synthesis during pupation. Small amounts of glucose and apparent glycogen are also present in the plasma of these insects. In *Bombyx* larval plasma there is also 0.04 to 0.12 gm. per 100 ml. of glucose-6-phosphate and smaller amounts of an apparent ketose phosphate.

We are grateful to Miss Margaret Hubbard for technical assistance and to Professor C. M. Williams for critically reading the manuscript.

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THE EFFECTS OF HYDROSTATIC PRESSURE UPON THE NORMAL AND NARCOTIZED NERVE FIBER*

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(Received for publication, January 28, 1957)

INTRODUCTION

Recently, the author reported the results of an investigation on the effects of hydrostatic pressure upon the electrophysiological properties of the vertebrate myelinated nerve fiber (1). In the present paper are reported further studies along this line. Two types of experiments will be presented: (*a*) experiments on the effects of hydrostatic pressure upon the electrophysiological properties of the giant axon of the squid, and (*b*) experiments demonstrating a dependence of narcosis of the axon upon temperature and pressure.

The preliminary experiments on these problems were conducted in collaboration with other members of this laboratory and were presented at the Fourth Symposium of the Society of General Physiology (2).

Methods

The Measurement of the Resting and Action Potential.—The giant axon of the stellar nerve of the squid, *Loligo pealii*, was used. The major portion of the small fibers around the giant axon was removed with a small pair of scissors under darkfield illumination. The methods of recording the resting and action potentials were similar to those used by previous investigators (3, 4). Two types of intracellular electrodes were employed for studying the action potential of the giant axon. Both electrodes were inserted into the interior of the axon through the cut end. One electrode consisted of a 25 μ insulated nichrome steel wire wound on a 100 μ insulated silver wire. The insulated silver wire was scraped bare for a total length equal to 10 to 20 mm. In the middle of the scraped area of the silver wire, the steel wire was scraped bare for a length equal to 1 to 2 mm. The nichrome wire was used for recording the action potential and the silver wire was used for stimulation. A precaution was taken to avoid direct metallic contact between the two wires. The second type of intracellular electrode used was a glass tube (50 to 100 μ in diameter) filled with isotonic KCl solution. This electrode was used for recording both the resting and the action potential. The axon was stimulated near the cut

* The work presented in this article was carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts.

end at a point several centimeters away from the tip of the pipette with two steel wire electrodes. The indifferent external electrodes with both methods of recording action and resting potentials were of the Ag–AgCl type. The method for recording the resting and action potentials with internal metal wire electrodes is illustrated in Fig. 1.

The Measurement of the Membrane Impedance during Activity.—The metal wire internal electrodes described above were used. The fiber was stimulated either through the long metal wire electrode in the axon or with a pair of external steel wire electrodes. The long internal electrode and the external Ag–AgCl electrode were connected to an arm of an A.C. impedance bridge. The ratio arms of the bridge were a pair of fixed resistors (one 10 ohms and the other either 100 or 300 ohms). The bridge was balanced

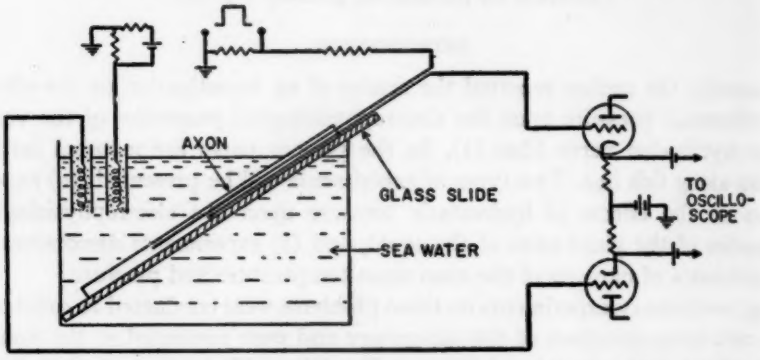

FIG. 1. Arrangement for recording action potentials using two internal metal wire electrodes. Axon was fixed to plate. Long metal wire (uninsulated for 10 to 20 mm.) was used for stimulation. Short metal wire (uninsulated for 1 to 2 mm.) was used for recording the action potential.

by varying the parallel capacity (0 to 200 $\mu\mu$f.) and the resistance (0 to 300 kilohms) in the third arm of the bridge. The frequency of the bridge A.C. was 10 to 20 kc. per sec. The output of the bridge, after filtering and amplification, was led to one beam of a dual channel oscilloscope. To the other beam was led the unfiltered response as recorded with the short internal electrode. In this manner simultaneous measurements were made of the membrane potential and impedance during activity.

The Measurement of the Time Constant of the Membrane.—The intracellular metal wire electrodes were used. The long internal electrode was used for passing a current pulse through the axonal membrane and the short electrode for recording the change in membrane potential brought about by the current flow. Two methods were employed for measuring the time constant of the membrane. In one method a long weak rectangular current pulse was passed through the membrane; the initial rate of change in membrane potential was a function of the membrane capacity and the plateau level of the potential was a function of the membrane resistance. In the other method a brief strong rectangular pulse of inward current was applied to the membrane charging its capacity. The extent of the initial rise in potential was an index of the mem-

brane capacity and the rate of exponential decay of the potential, an index of the time constant of the membrane.

The Measurement of the Threshold Membrane Current.—The threshold membrane current was determined using the arrangement of Fig. 1. The long metal wire was used for passing the test current pulse through the membrane. The short metal wire was used for recording the response.

The Measurement of the Conduction Velocity.—The axon was placed inside a plexiglass tube 30 to 40 mm. long and about 1 to 1.5 mm. inside diameter. At one end of the tube the axon was in contact with two platinum wires that were used for stimulation. The axon mounted in the tube was immersed in sea water. In this manner direct contact with the mineral oil was avoided (*cf.* reference 1). An estimate of the conduction velocity was obtained from the shock response interval.

Variation of Pressure.—Hydrostatic pressure was varied from 14.7 to 16,000 psi. The reaction vessel employed was of the "outside cap compression closure type." (For more details of the assembly for increasing the pressure and of the need for surrounding the fiber with large volumes of aqueous solutions to prevent heat change: *cf.* reference 1.)

The Temperature.—The results to be described were obtained at 10°–15°C. unless special mention is made.

Other Instruments.—Other instruments employed included a General Radio Company beat-frequency oscillator, a Spencer-Kennedy variable electronic filter, a DuMont dual beam cathode ray oscillograph, a Grass kymograph camera, a Grass stimulator, a Tektronix stimulator, and a Tektronix preamplifier. The cathode follower utilized a Z729 Emitron tube.

RESULTS

The study of the effects of pressure upon the electrophysiological properties of the giant fiber was complicated by the finding that at pressures of 3000 to 7000 psi the fiber fired spontaneously. Reliable measurements of most properties could be made only at pressures below the threshold for spontaneous discharge.

Resting Potential.—In the range of 14.7 to 5000 psi the resting potential as determined with the internal glass pipette was found to be independent (within 5 per cent of pressure.

Membrane Resistance and Capacity.—At pressures above atmospheric but below 4000 to 6000 psi the resistance of the membrane was slightly increased (Fig. 2). At 5000 psi the resistance was 10 to 20 per cent higher than at 14.7 psi. At pressures above 4000 to 6000 and below 12,000 psi the membrane resistance often decreased to as much as one-half to one-third the resistance at 14.7 psi. Under these conditions the recovery of the original resistance upon decompression was poor. These changes in the membrane resistance are shown by the variations in the plateau level of the membrane potential in Fig. 2. Since the intensity of the rectangular current pulses applied through the membrane was held constant, the change in the (D.C.) potential level was a direct measure

of the membrane resistance. In the record of Fig. 2, the change in the membrane potential caused by the current pulse was (before any compression) about 45 mv. Essentially the same results were obtained in the experiments in which the current intensity was reduced so that the potential variation was within a few millivolts below or above the resting potential (recorded after higher amplification).

In the range of 14.7 to 6000 psi, the membrane capacity was found to be independent of pressure. This is shown by the coincidence of the initial rate of potential fall in the two lower traces in Fig. 2. A change in capacity over

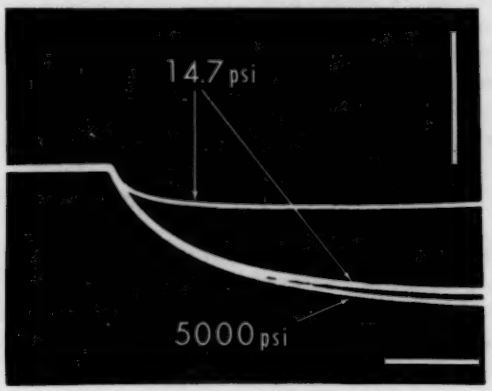

FIG. 2. Records showing the effect of hydrostatic pressure upon the membrane resistance and capacity. The membrane potential was modified by passing a constant current through the membrane using the arrangement of Fig. 1. Middle tracing at 14.7, lower tracing at 5000 and upper tracing at 14.7 immediately after subjecting the axon to 12,000 psi. Vertical bar at right subtends 50 mv. The duration of the current pulse was 20 msec. Pulse amplitude 3×10^{-6} amperes. Time marking 5 msec.

20 per cent would have been detected. Even after the membrane resistance suffered an irreversible reduction under high hydrostatic pressure, it was not possible to detect a measurable change in the membrane capacity.

Threshold Membrane Current.—The threshold membrane current and the threshold membrane potential were found to be significantly dependent upon pressure. The threshold membrane current as well as the threshold membrane potential decreased with an increase in pressure. A decrease in threshold was apparent at pressures below 100 psi. At pressures from 3000 to 7000 psi the threshold approached zero, and the fiber started to discharge nerve impulses spontaneously.

Conduction Velocity.—The conduction velocity was found to be only slightly affected by pressure. At 5000 psi the conduction velocity as evidenced by the shock response interval was decreased by about 5 to 15 per cent.

Action Potential.—At 5000 psi the amplitude of the action potential was found to be relatively unaffected. The duration of the falling phase of the response was markedly increased by an increase in pressure. At 4000 psi the duration was increased by about 40 to 60 per cent. The duration of the rising phase was also increased though always with a considerably lower coefficient than that of the falling phase. At 4000 psi it was increased by about 20 to 35 per cent. The effects of pressure upon the action potential are illustrated in Fig. 3. The record at the left was obtained at 14.7 psi and that at the right at 5000 psi. The duration of the rising phase was increased by about 35 per cent and that of the falling phase by about 65 per cent; there was no appreciable change in the amplitude of the response.

Membrane Impedance during Activity.—With the present system of recording

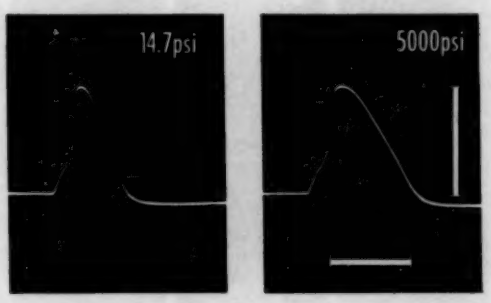

FIG. 3. Membrane action potentials of a squid giant axon recorded at 14.7 psi and 5000 psi. The arrangement of Fig. 1 was used. Vertical bar at right subtends 100 mv. Time marking 5 msec.

simultaneously the action potential and the membrane impedance loss, it was found that the peak of the impedance changes followed the peak of the potential changes by 0.5 to 3 msec. depending upon temperature and pressure. At 5000 psi the amplitude of the maximal bridge unbalance was in most experiments decreased by about 15 per cent or less. At the same pressure the duration of the bridge unbalance was increased by a factor of about 1.7 to 2.0. The transition from the unbalanced state to the balanced was very gradual so that an accurate determination of the duration of the changes in impedance was not possible. For this reason the pressure coefficient of the duration given above should be considered as being somewhat arbitrary. The impedance measurements were carried out at 5°–10°C.

The Temperature and Pressure Dependence of Drug Action.—Application of 3 to 7 per cent ethanol–sea water at room temperature (20°–23°C.) reduced the amplitude of the spike by about 20 to 50 per cent. Occasionally, under alcohol, the response obtained (when stimulated with an internal electrode)

varied with the intensity of the stimulus. When the temperature of the narcotized axon was lowered to 6°–10°C. the amplitude of the action potential was almost completely or partially restored. A restoration of the heightened threshold was also observed at low temperatures. In those instances in which the response of the alcohol-treated axon was not all-or-none at room temperature, lowering of the temperature restored the all-or-none property.

The temperature dependence of narcosis in the giant axon is illustrated in Fig. 4. In this experiment two metal wires were inserted into the interior of the axon, as shown in Fig. 1. One wire was used for stimulation and the other

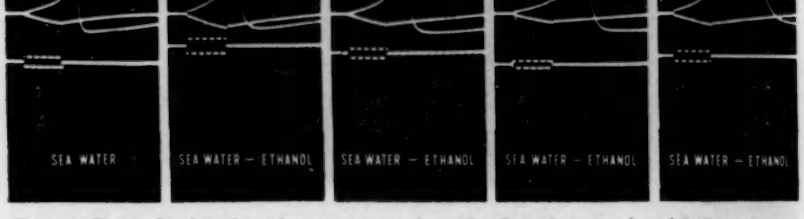

FIG. 4. Records showing the counteraction of ethanol narcosis with low temperatures. The amplitude of the response of the giant axon treated with ethanol at 22°C. varied with the intensity of the stimulus. At 4°C. the fiber fired spontaneously. The record in this figure (at 4°C.) was obtained by electrical stimulation with the internal wire electrode. The arrangement of Fig. 1 was used. Concentration of ethanol 3 per cent. Time marking 5 kc.

for recording the action potential. The record at far left (obtained at 22°C. in normal sea water) is the control. The second record from the left was obtained at 22°C. in sea water containing 3 per cent ethanol. The response obtained was not all-or-none. In the record presented in the figure the peak of the change in potential was reached following the end of the stimulating pulse. Under the same conditions action potentials of a much higher amplitude were obtained with higher stimulus intensities. The three records at the right were obtained at low temperatures (17°, 10°, and 4°C.) in the presence of ethanol in the sea water. At these low temperatures the response was larger and all-or-none.

The increase in the amplitude caused by lowering the temperature was far greater in the narcotized than in the unnarcotized axon. For a drop in tempera-

ture from 22° to 4°C. the amplitude of the response of a normal fiber increases only by about 5 to 10 per cent. For the same decrease in temperature, the amplitude of the response of the narcotized fiber indicated in Fig. 4 was almost doubled. The temperature coefficient of the duration of the response of the ethanol-treated axon is approximately the same as that of the unnarcotized axon.

Application of high pressures (3000 to 7000 psi) upon a narcotized axon had an effect similar to that of low temperatures, though not as dramatic. The effects of hydrostatic pressure upon ethanol narcosis are illustrated in Fig. 5. In this experiment a glass pipette was introduced into the interior of the giant axon through the cut end. Near the cut end the axon was stimulated with two steel wires. The response recorded through the tip of the glass pipette was

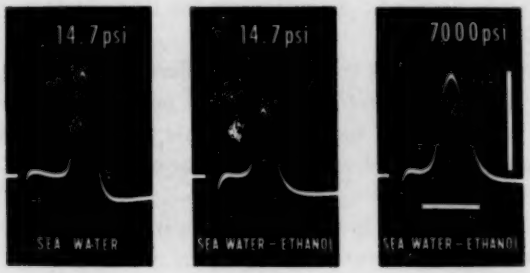

FIG. 5. Records showing counteraction of ethanol narcosis with high pressure. A glass pipette internal electrode was used. The axon was stimulated near its cut end with two steel wires. The glass pipette recorded the conducted response. Ethanol concentration 3 per cent. Temperature 22°C. Vertical bar at right subtends 100 mv. Time marking 1 msec.

a conducted response. The record at left represents the response that was obtained at 14.7 psi in normal sea water. The response shown in the middle record was obtained at 14.7 psi in sea water containing 3 per cent ethanol. The response shown in the record at the right was obtained at 7000 psi in ethanol–sea water. Although the amplitude was increased appreciably, it was still slightly subnormal.

DISCUSSION

In general the effects of pressure upon the giant axon are similar to those upon the myelinated nerve fiber. In both types of fibers one of the most marked effects of pressure involved the duration of the response. In both types of fibers the conduction velocity and the amplitude of the response were relatively unaffected by pressure. A marked reduction in threshold and a spontaneous discharge of nerve impulses were two effects of pressure observed only in

the giant axon. In the myelinated nerve fiber at high pressures the rheobase was only slightly affected and no spontaneous firing was encountered.

Most of the effects of high pressures upon narcosis and upon the electrophysiological properties of the fiber are similar to the effects of low temperatures (for review *cf.* reference 2). The temperature dependence of the pressure effects upon the nerve fiber is under investigation.

A pronounced temperature and pressure dependence of drug action in a variety of chemical and biological systems is well known (for review *cf.* reference 5). An example of this phenomenon is the counteraction by low temperatures or high pressures of narcosis in whole animals. It was very surprising to find that this problem had not been investigated either on the nerve fiber or on the nerve trunk. The experiments presented in this paper on the temperature and pressure dependence of drug action may provide the basis for an explanation of low temperature or high pressure counteraction of some types of narcosis in animals.

The approach of measuring a process at different temperatures, pressures, pH, and concentrations of inhibitors has been used extensively in the study of a number of chemical and biological systems (5). Perhaps further studies on the nerve fiber may prove of some value in the elucidation of the mechanism of excitation.

The sensitivity of the axon to pressure makes it feasible that similar or different properties of other elements in the nervous system may also be sensitive. This in turn would indicate that the *direct* effects of pressure on processes in the nervous system should not be overlooked in the consideration of problems of underwater high pressure physiology.

SUMMARY

The properties of the giant axon of the squid *Loligo pealii* were studied at different hydrostatic pressures from 14.7 to 16,000 psi. At 4000 psi the resting potential, the membrane resistance, membrane capacity, the conduction velocity, the amplitude of the action potential, and the maximal change in the membrane impedance during activity were only slightly affected. At the same pressure the duration of the falling phase of the action potential was increased by about 40 to 60 per cent and the duration of the rising phase by about 20 to 35 per cent. The duration of the membrane impedance change during activity was increased by 50 to 100 per cent at 4000 psi. At pressures even slightly above atmospheric the threshold membrane current was appreciably reduced. At about 3000 to 7000 psi the fiber fired spontaneously. At pressures considerably above 5000 psi the membrane resistance decreased to about one-half to one-third the original value. The narcotizing effect upon the nerve fiber of 3 to 7 per cent ethanol was partly or almost completely opposed by low temperatures or high pressures.

The author wishes to acknowledge the help of Dr. I. Tasaki, Dr. S. Hagiwara, and Mrs. N. Tasaki in the work presented in this article.

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DEMONSTRATION OF TWO STABLE POTENTIAL STATES IN THE SQUID GIANT AXON UNDER TETRAETHYLAMMONIUM CHLORIDE*

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(Received for publication, February 5, 1957)

During the course of experiments designed to investigate the effects of various chemicals injected into squid giant axons (2), it was found that quaternary ammonium ions are capable of prolonging the duration of the action potential without affecting the properties of the resting membrane appreciably. A similar effect of quaternary ammonium ions upon the action potential of other excitable tissues has been reported by several investigators (8, 11, 18). Following a longitudinal injection of a proper amount of tetraethylammonium chloride (TEA) into the whole length of a giant axon, the configuration of the normal action potential undergoes a rapid change into a response with an initial peak followed by a long plateau. The response of the squid axon under TEA resembles in many respects the action potential of the cardiac muscle (28–30).

The present paper describes the results of an electrophysiological investigation of the mechanism of action potential production in the giant axon membrane under the influence of TEA. The main technques employed are similar to those developed by Marmont (20), Cole (4), and Hodgkin *et al.* (16) Inserting long metal wire electrodes longitudinally into the axon, either the membrane potential or the membrane current was controlled and the changes in the membrane properties were examined under various experimental conditions. The results obtained are interpreted as supporting the view that there are two stable states in the membrane and that the process of initiation and abolition of the action potential (26) represents a transition of the membrane between these two stable states.

METHOD

1. Dissection of Giant Axons.—Giant axons were dissected out of the North Atlantic squid, *Loligo pealii*, available at the Marine Biological Laboratory in Woods

* The work presented in this article was carried out at the Marine Biological Laboratory, Woods Hole.

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Hole by the technique described by previous investigators (5, 16). The axon was carefully, though not extensively, cleaned under darkfield illumination by removing the major portion of the small nerve fibers with a pair of small scissors. The diameter of the axons used was usually between 0.55 and 0.35 mm.

2. Longitudinal Injection of TEA.—The axon was mounted horizontally across a glass plate 34 mm. wide and its tied ends were stretched beyond the edge of the plate. The tied ends were fixed to the lucite platform to which the glass plate was glued. With a pair of small scissors, two small incisions were made in the axon membrane, one at each edge of the glass plate. Through one of the holes made by the incision, the injection pipette of 70 to 100 μ in outside diameter was inserted along the axis of the axon. In the experiment of Fig. 3, the recording electrode was a glass pipette of approximately 60 μ inserted into the axon through the hole at the other edge of the glass plate. Injection was started when the tip of the injection pipette was near the end of the axon on the side opposite to the point of insertion. While the deeply stained solution of TEA was flowing out of the injection pipette at a uniform rate, the pipette was withdrawn at a uniform velocity. The details of the procedure of injection and precautions to be taken during this procedure will be discussed elsewhere (2). In the later experiments (Fig. 4 and others), an assembly of metal electrodes was introduced into the axon after the injection pipette had been withdrawn.

3. Preparation of TEA Solution.—In most of the experiments described in this paper, an isosmotic (0.55 M) solution of tetraethylammonium (TEA) chloride was prepared by diluting a commercially available drug, etamon (Parke, Davis & Company). The effect of this drug was exactly the same as that of the sample kindly supplied to us by Dr. Lorente de Nó. The solution was stained with chlorphenol red and then its pH was adjusted to 6.6–6.7 by adding a trace of 0.5 M KOH solution.

The total amount of the TEA solution injected into an axon was between 0.6 and 1.2 mm^3. Since the length of the injected portion of the axon was approximately 30 mm., the final concentration of TEA in the axoplasm was estimated to be between 40 and 60 mM. When a larger amount of the TEA solution was introduced into the axon, there was always an outflow of the stained TEA solution through the cut end of the axon.

4. Recording of the Membrane Potentials.—A glass pipette electrode of 60 to 100 μ in diameter filled with an isosmotic KCl solution was used to record both the resting and action potentials internally. A lightly chlorided silver wire of 100 μ in diameter, immersed in the fluid in the pipette, served to connect the pipette to the input of a cathode follower. A British tube, Z729, operated in triode connection, was used as a cathode follower. Its input capacity was of the order of 5×10^{-12} farad. A precaution was taken, therefore, not to use a recording electrode with a resistance exceeding a few megohms.

When recording of the resting membrane potential was not required, either a bare silver wire of 100 μ diameter or a nichrome-steel wire of 20 μ diameter was used to measure transient changes in the membrane potential. The reliability of the nichrome-steel wire electrode for this type of potential measurement has been demonstrated previously (27).

5. Measurement of the Membrane Impedance during Activity.—The time course of the change in the membrane impedance associated with an action potential was

determined with the arrangement schematically illustrated in Fig. 1. An alternating current Wheatstone bridge was constructed with two fixed resistors, r_1 and r_2, a variable resistance ($0 \sim 0.2$ MΩ) with a parallel capacity (up to 200 μμfd.), and with electrodes in and outside the axon membrane. The internal impedance electrode was made with an enamelled silver wire, the enamel of which was removed for a length of 10 mm. at the tip. The bare portion of the wire was inserted in the middle of an axon of 34 mm. length. The resistors in the ratio arms of the bridge, r_1 and r_2, were 10

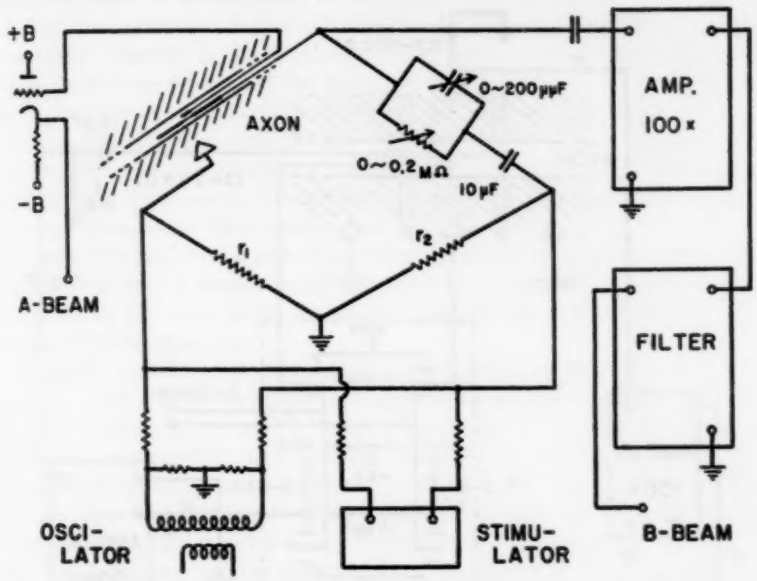

FIG. 1. Diagram illustrating the impedance bridge used to record the time course of the impedance loss during activity. The long impedance electrode was a metal wire with 10 mm. long exposed surface. The potential electrode had 1 mm. long exposed surface. Resistances r_1 and r_2 were 10 and 100 (or 200) ohms respectively. Further explanation in text.

and 100 ohms (or 10 and 200) respectively. The bridge A. C. was usually 20 kc./sec., in some cases 10 kc./sec., and was supplied by a beat-frequency oscillator (General Radio, type 1304-A). The A. C. voltage across r_1 was not allowed to exceed about 30 mv. peak-to-peak.

The bridge unbalance was amplified 100 times with a preamplifier (Tektronix, type 122) and then filtered by a variable electronic filter (Spencer-Kennedy Laboratory, Model 302) which was adjusted to pass a narrow band of frequencies around the bridge A. C. frequency. The output of the filter was led to one channel of a dual beam oscilloscope (DuMont, type 322). The ringing in the filter circuit following a short transient pulse decayed at a rate of approximately one-third per cycle at 20 kc./sec.

To record action potentials of the axon simultaneously with the membrane im-

pedance, an independent metal wire electrode (20 μ steel wire) was inserted into the axon together with the impedance electrode. Direct metallic contact between the two internal electrodes was prevented by using enamel and De Khotinsky cement. The membrane potential was recorded at the middle of the bare portion of the impedance electrode. In later experiments, action potentials were recorded through the impedance electrode. Stimulating current pulses from a stimulus isolation unit (Grass Co., Model SIU-4A) were delivered to the axon through the impedance electrode.

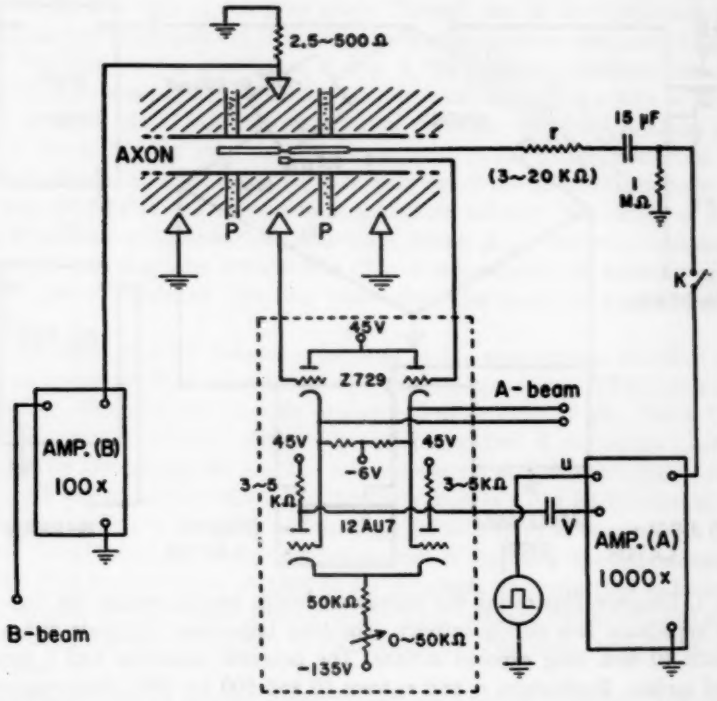

FIG. 2. Diagram showing the arrangement used for voltage clamp experiments. The thick portions of the electrodes indicate the exposed surfaces of the metal wire electrodes. The portion of the axon between the two partitions was approximately 9 mm. long. Further explanation in text.

Under these circumstances, the axon membrane is partly short-circuited by the variable resistance. Because of the blocking condenser (10 μfd.) connected in series with the variable resistor and condenser, the distortion of the recorded action potential by the bridge was not very serious. A slightly disturbing artefact arising from this circuit was the complication caused by the stimulating current charging the variable condenser in the diagram. When the voltage pulse from the stimulus isolation unit comes to an end, the axon membrane is subjected to a short current flowing in the reversed direction. Because of this artefact, it was somewhat difficult to abolish the action potential with the arrangement of Fig. 1.

With this impedance bridge, a considerable difficulty was encountered when an attempt was made to determine the absolute values of the membrane capacity and resistance. At 20 kc./sec. the A. C. voltages at the two outputs of the oscillator were not exactly in opposite phase. There were stray capacities which prevented the bridge from operating under simple, ideal working conditions. Unless the frequency of the bridge A. C. was lowered below about 5 kc./sec., it was not possible to correctly determine the capacity and the resistance in the unknown arm from the readings in the known arms. In the present paper, therefore, the method of impedance measurements was used to obtain more or less qualitative information as to the conductance of the membrane during activity, the bridge unbalance being considered as a monotonic function of the membrane conductance. A simple calculation of the membrane impedance shows that the bridge unbalance should be proportional to the change in the membrane conductance except at or near the peak of activity.

6. *Voltage Clamp.*—The arrangement diagrammatically shown by Fig. 2 was employed to measure the membrane current necessary to "clamp" the membrane potential along a rectangular time course. A giant axon was mounted horizontally on a glass plate as in the other experiments. The glass plate used in this experiment was, however, provided with a pair of partitions (*P* in the figure) made with pieces of lucite plates and with a small amount of vaseline. An assembly of metal wire electrodes similar to that used in the impedance measurement was introduced into the axon as shown in the diagram.

A rectangular voltage pulse from a pulse generator (Tektronix, type 161) was led into one of the two inputs of a differential amplifier (Tektronix type 122) which had a flat response between 40 kc. and 0.1 C.P.S. The output of the amplifier (Amp. A in the figure) was connected to the long metal wire electrode in the axon through a key (*K*), a condenser (15 μfd.), and a resistor (3 to 20 kilohms). The polarity relationship between the input and the output of the amplifier was such that when the potential at the input was rapidly raised (*i.e.* $u > 0$), there was a sudden flow of current from the electrode in the axon into the axoplasm. Such a sudden flow of an outward current should result in a sudden rise in the membrane potential which eventually transmitted to the second input of the differential amplifier (Amp. A). The cathode follower stage, indicated by a square box of a dotted line, was designed simply to transmit the potential difference across the axon membrane to the single-ended output of the stage. The principle of voltage clamping by this arrangement is as follows:—

In the steady state during which the potential (u) at the input of the differential amplifier (A) is maintained at a certain positive level, there is a continuous flow of an outward current through the axon membrane which maintains the membrane potential (V) approximately at the potential of the input of the amplifier (A). Denoting the voltage from the pulse generator by u, the gain of the differential amplifier (A) by n, the resistance between the output of the amplifier and the internal electrode by r, and the variation in the membrane potential by V, one finds that in the steady state the membrane current I is given by the expression

$$I = \frac{n(u - V) - V}{r}$$

(The gain of the cathode follower stage is assumed to be unity.) If the value of $(V + Ir)/n$ is negligibly small compared with V or u, therefore, V should be equal

to u. The current I varies with the membrane resistance R and with any change in the E. M. F. of the membrane. If one assumes for simplicity that the E. M. F. of the membrane stays constant during the period of voltage clamp, then $V = IR$. Introducing this relation into the above expression for I, one obtains the relation

$$I\left(R + \frac{R}{n} + \frac{r}{n}\right) = u.$$

Since n is 10^3 and r is about 5×10^3 ohms in the present experimental conditions, the term r/n in the parenthesis is equal to 5 ohms. As long as R (the resistance of the axon membrane between the two partitions) is far greater than 5 ohms, IR should be approximately equal to u no matter how R varies during the voltage clamp. For a portion of the axon of 8 to 9 mm. length, the resistance R of the membrane under TEA undergoes a transient reduction from the resting value of 15 to 20 kilohms down to 300 to 500 ohms when V is about 80 mv. (see section 4 under Results). The membrane potential V should, therefore, diverge from u by 1 to 2 per cent. Actually there is a rise in the E. M. F. of the membrane when R goes down; this situation tends to reduce the divergence considerably.

In a normal axon, *i.e.* in an axon without injection of TEA, the membrane resistance undergoes a greater reduction during activity. Consequently a small variation in the membrane potential was observed when an attempt was made to clamp the membrane at a potential slightly higher than the ordinary threshold level. A correction was made for this small potential variation by superposing a small double condenser pulse on the square pulse of u. By adjusting the size and the delay of this double condenser pulse, it was always possible to vary the membrane potential along a rectangular time course.

The membrane current required for clamping the potential was determined by recording the potential drop across a small resistor (2.5 to 500 ohms) connected between the sea water around the axon and ground. By choosing a proper value for this resistor, the potential drop across this resistor was restricted to a range not exceeding a few millivolts. The currents flowing through the internal current electrode near its ends did not enter into the record because of the two lateral electrodes grounding the lateral pools of sea water independently.

A precaution has to be taken with this circuit to prevent an oscillation in the amplifier. In practice a high-frequency oscillation was found to start only (*a*) when the resistance between the axon and the external electrodes was unduly raised by reducing the amount of sea water around the axon, or (*b*) when the electrical contact between the potential electrode and the axoplasm became poor. Another precaution to be taken is to adjust the D. C. output voltage of the differential amplifier (A) carefully to zero; otherwise there will be a transient change in the membrane potential when the contact K is closed. With this arrangement it is not possible to clamp the resting potential of the membrane at new artificial levels.

RESULTS

1. Prolongation of the Action Potential by TEA.—An internal recording electrode, a glass pipette in this series of experiments, was thrust down the

axis of a giant axon until the recording tip reached the point half-way between the two ends of the axon. Then, the injection pipette was introduced into the axon through a hole at the other end of the axon. The tip of the injection pipette was pushed beyond the recording electrode so that a considerable portion of the two pipettes overlapped in the axon. This procedure was not difficult when the diameter of the axon was around 500 μ. Since both of the pipettes were slightly smaller than 100 μ, it was possible to maneuver these pipettes in the middle of the axon without touching the membrane. Stimulating shocks were applied to the axon near one of the ends of the axon, usually on the side where the injection pipette was introduced.

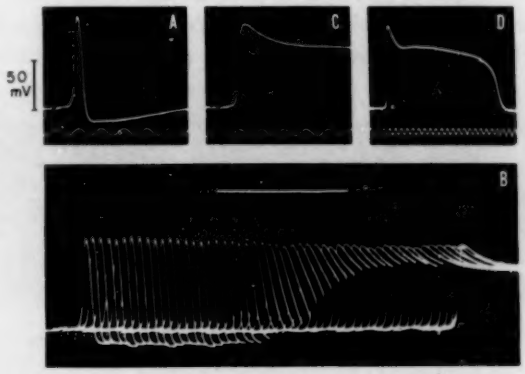

FIG. 3. Records showing changes in the time course of the action potential caused by intracellular injection of tetraethylammonium chloride. Record A, before injection. The white line in record B represents the time during which the injecting pipette was moved from one end of the axon to the other; shock interval, 1 sec. Records C and D, after injection. Time marker, 1 msec. 22°C.

Record A in Fig. 3 was obtained when the two pipettes were in the axon (but before the injection of TEA). The size and shape of this action potential are similar to those obtained by previous investigators (6, 12). Record B was taken with a continuously moving film, while the axon was being stimulated at a frequency of 1 shock per sec. The bar in this record indicates the period during which the TEA solution was injected into the axon. During this period the injection pipette was moved toward the point of insertion at a uniform velocity.

Within a few seconds after injection of the TEA solution into the space around the recording electrode, the duration of the response was found to start to prolong. As can be seen in the figure, TEA did not change the resting potential of the axon. A marked prolongation of the spike duration was often associated with a slight (5 to 10 per cent) reduction in the spike height. Records C and D are single-frame photographs taken after injection.

After rapid prolongation of the spike duration following an injection of TEA, the spike showed a slight tendency to a further prolongation. Then, a steady state was reached and the spike remained unchanged for more than several hours. The spike duration in the steady state depended upon the amount of TEA injected into the axon; a small amount of TEA brought about a small change in the response, and too large an amount reduced the spike height without producing a profound prolongation. In the following experiments, an injection of 0.6 to 1.2 mm.^{3} of isosmotic TEA solution into a 30 mm. length portion of an axon of 500 μ diameter was chosen as a standard procedure. This procedure altered the normal spike of about 0.7 msec. duration at room temperature (22°C.) into a response of 10 to 30 msec. duration.

Besides a marked increase of the total spike duration, TEA brought about a conspicuous change in the configuration of the response. Following a quick

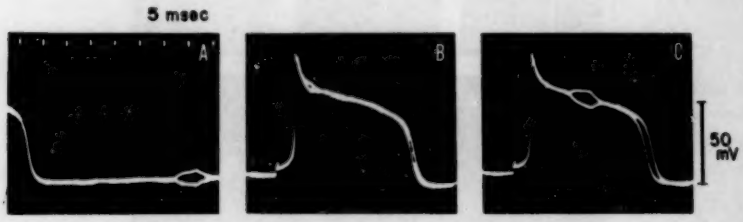

FIG. 4. Effect of long test pulses of constant current upon the time course of the membrane potential. The pulses were applied through a long internal electrode and the membrane potential was recorded in the middle of the long internal electrode. In each record two sweeps are superposed.

fall in the membrane potential from the peak, the potential began to fall at an extremely small rate, forming a "plateau." This plateau was abruptly terminated by a "shoulder," the potential level of which varied considerably with the amount of TEA injected. There was, as a rule, an "undershoot" of potential following the end of the spike potential. Evidently, there is a striking similarity between the action potential of the squid giant axon under TEA and that of the cardiac muscle (7, 17, 28–30). When the squid axon under TEA was cooled down to 3°C., the spike duration was found to increase up to 500 msec. or more. Under such circumstances, a record of a squid action potential would be almost indistinguishable from that of a cardiac muscle (*e.g.* Fig. 8E in reference 29). It will be shown in the following sections that this similarity between the squid axon under TEA and the cardiac muscle is by no means superficial.

When the TEA solution was introduced in one-half of the whole length of the axon, it was possible to show, by moving the recording electrode along the axis of the axon, that the response has a normal duration in the normal region

and a tremendous duration in the injected region. Near the boundary between the normal and injected regions, a mixture of long and short responses was obtained. Such preparations often showed repetitive firing of responses, resulting probably from an interaction between prolonged and normal responses.

We have not examined the effect of TEA injected at one spot of an axon as in the experiments by Grundfest, Kao, and Altamirano (10). However, it appears to us certain that such a localized injection of TEA would not effectively prolong the spike duration, because, as we shall see in the following sections, the normal responses from the neighboring region would "abolish" the response from the injected region.

It is interesting to note that various mixtures of the TEA solution and sea water up to one to one ratio applied externally to the squid giant axon (for 1

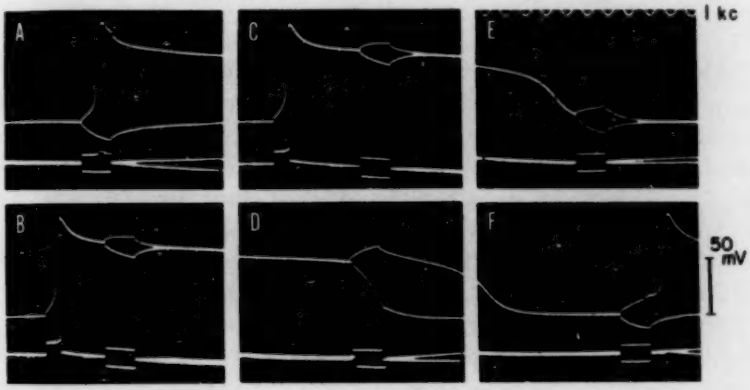

FIG. 5. Same as Fig. 4, except that the current pulses were shorter and stronger. The lower trace indicates the time course of the membrane current.

hour or more) did not bring about any appreciable change in the duration of the recorded action potential.

2. Resistance of the Axon Membrane during Activity.—The similarity between the response of the squid axon under TEA and that of the cardiac muscle became more impressive when the membrane resistance during activity was compared in the two systems. The resistance measurements on the squid axon membrane were made with two internal metal wire electrodes inserted into the axon as shown in Fig. 1. When the test pulse method was employed to estimate the membrane resistance, the long current electrode was connected to a pair of square pulse generators through two separate 1 megohm resistors. Recordings were made through the other metal wire electrode which had its exposed surface of 1 to 2 mm. long in the middle of the current electrode. A limitation of this method of measuring the membrane resistance was that the

intensity of test pulses has to be kept at a low level in order to avoid initiation or "abolition" of responses.

Fig. 4 shows an example of the records obtained by using subthreshold test pulses. The current pulses delivered during the plateau of the action potential (record C in the figure) brought about a potential variation which was nearly as large as (or slightly greater than) that observed a long period of time after the end of the action potential (record A). At the peak of the action potential, the potential variation caused by a current pulse of the same intensity was definitely smaller, indicating that the membrane resistance was very low at that moment.

This property of the membrane during activity is very similar to that of the vertebrate cardiac muscle (Fig. 16 in reference 29), and also to that of the crustacean muscle fiber treated with tetrabutylammonium ions (Fig. 13 in reference 8). In both of these muscle fibers, there was an apparent increase in the membrane resistance toward the end of the action potential. This has also been shown to occur in the squid axon under TEA.

Record D in Fig. 5 shows the effect of current pulses applied near the end of the action potential prolonged by TEA. The potential variation caused by the pulse of an outward current (shown by the upward deflection in the figure) has a size similar to that of the potential changes produced by inward current pulses in records A, C, E, and F. It is seen that the pulse of an inward current in record D brought about a disproportionately large potential variation. As has been pointed out by Fatt and Katz (8), this anomalous behavior of the membrane at the end of an action potential is an indication of a premature termination or abolition of the response and does not necessarily indicate a high membrane resistance at this moment. Similarly, the large effect caused by outward current pulses in records E and F is undoubtedly attributable to the tendency of the axon membrane to respond to the test pulse with a second action potential. The slightly smaller potential variation seen in record B is an indication of a smaller membrane resistance at this moment.

The results obtained by the impedance method (Fig. 1) are consistent with those described above. In Fig. 6, the records in the left column were taken from a normal; *i.e.*, non-injected, axon. The upper record was taken with the bridge balance for the impedance of the axon at rest. The lower record was obtained by adjusting the bridge in such a way that the best balance was observed at the peak of the action potential. The temporal relationship between the impedance loss and the membrane potential in these records is similar to that reported by Cole and Curtis (5).

The records in the middle column were taken from an axon into which the TEA solution had been injected. In spite of the tremendous difference in the falling phase of the action potential between the normal and the TEA-treated axons, the time course of the impedance loss during activity was not very

different in the two axons. The maximum impedance loss was less in the TEA-treated axon than in the normal. The quantitative aspect of this difference, however, was worked out by the method of voltage clamp (section 4) and not by the method of impedance measurement.

The records in the right-hand column in Fig. 6 were taken with a slower time base at a lower bridge frequency. Although the time resolution was re-

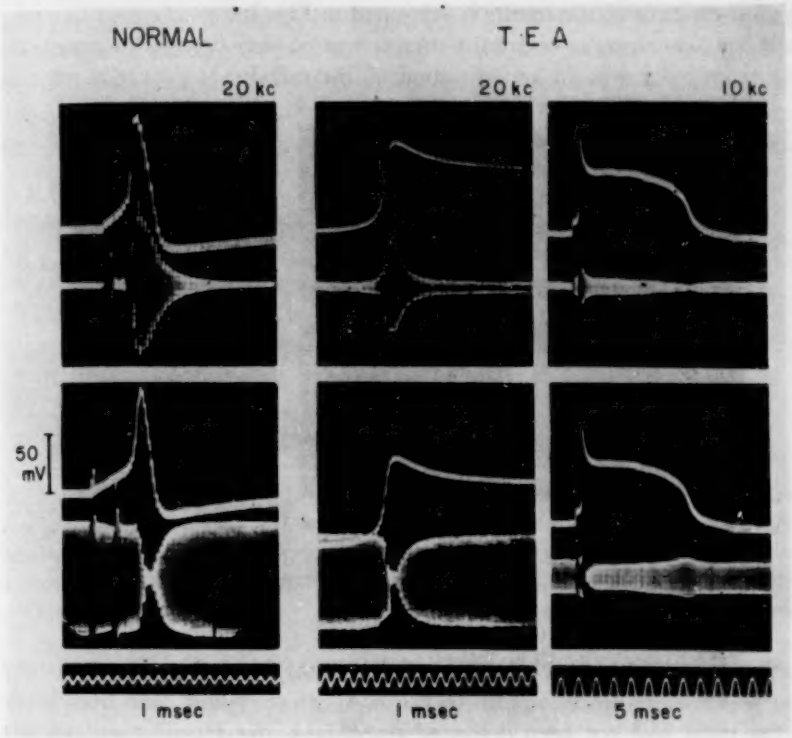

FIG. 6. Simultaneous recording of the action potential and the membrane impedance in a normal axon (left) and in the same axon treated with tetraethylammonium chloride (right).

duced by lowering the bridge frequency, the sensitivity of the bridge to slow impedance changes was greatly enhanced. In these records the unbalance at the peak of the action potential was so large in amplitude that it was not discernible. It is seen in these records that during the falling phase of the action potential there was a gradual return of the membrane impedance toward the normal. At about the shoulder of the action potential, the membrane impedance rose slightly above the resting value.

3. Abolition of Action Potentials.—The electrode arrangement used to demonstrate abolition of action potentials in the squid axon was the same as that for the experiments of Figs. 3 and 4. Through the long current electrode, a stong, short pulse of inward or outward current was applied to the membrane to produce a sudden change in the membrane potential during the course of an action potential. The duration of the pulse was about 0.2 msec. or less; its intensity was adjusted to produce a potential variation of 10 to 100 mv. A typical example of the results is presented in Fig. 7.

When a weak pulse of inward current was passed through the membrane in the falling phase of an action potential, the membrane potential went down

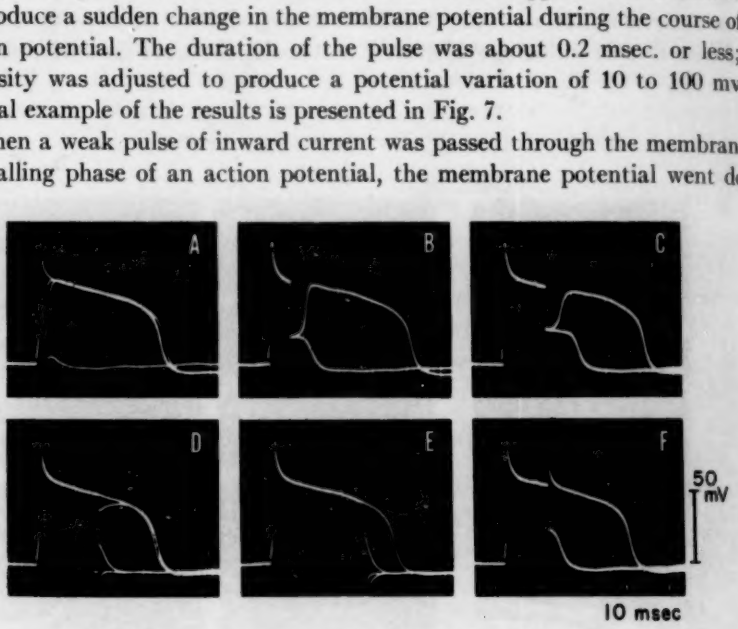

FIG. 7. Effect of short, strong current pulses applied during the course of action potential of a giant axon treated with tetraethylammonium chloride. Two to four sweeps were superposed on each record.

during the short period of current flow. Then, on termination of the pulse, the potential returned rapidly to the level which would have been attained if the pulse had not been delivered (records A and D). The amount of the potential change increased with the intensity of the current pulse. When the pulse intensity exceeded a certain value, the membrane potential did not rise after the end of the applied pulse, but instead it went down to a definite level which was independent of the applied shock. The premature termination, or abolition, of the action potential in the squid axon was found to be all-or-none, just as in the nodal membrane of the myelinated nerve fiber and in the cardiac muscle (26, 29).

When the pulse intensity was adjusted to the threshold for abolition, records as shown in Fig. 7 B and C were obtained. The membrane potential stayed at an approximately constant level for a variable length of time and then it went either up or down to reach one of the "two stable potential levels." This

variability in the time course of the membrane potential is analogous to that seen in initiating an action potential by a threshold stimulus. In record F the effect of a pulse of an outwardly directed current is shown together with that of an inward current of the same intensity.

It is interesting to note that the "threshold membrane potential" for abolition rises continuously during the course of activity. In the early phase of the action potential, the threshold potential was close to the ordinary threshold potential necessary for initiating an action potential (record A). As the membrane potential gradually fell, the threshold membrane potential for abolition rose continuously and approached the level of the "shoulder." With the abolishing pulse delivered near the shoulder as in record E, it was difficult to determine the threshold for abolition, because of the great liability to abolition

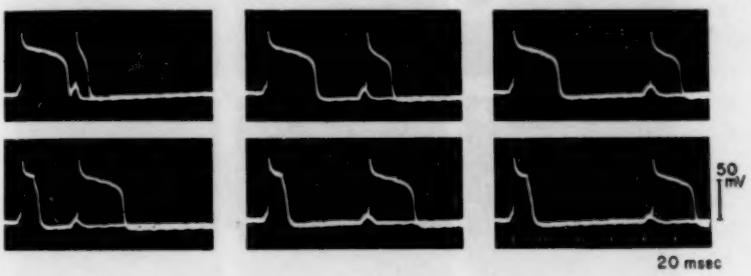

FIG. 8. Upper row, demonstration of refractoriness following a prolonged response of a squid giant axon treated with tetraethylammonium. The second, test shocks were adjusted to the threshold and two successive sweeps were superposed. Lower record, demonstration of absence of refractoriness after an abolished action potential of the same axon.

at this stage. These observations support the view that the natural termination of an action potential is the result of the impingement of the membrane potential upon the abolition threshold (26).

Another point of interest in Fig. 7 is the fact that there is an "undershoot" of variable sizes following abolition of an action potential. Following an unabolished action potential, the membrane potential stays at a level of 5 to 10 mv. below the level of the resting potential. When the action potential was abolished in its later phases (records D and E), it was found that the lower potential level which was attained by the membrane following abolition was close to that of the normal undershoot. As the abolishing pulse was delivered in the earlier phases, the lower stable potential level gradually approached the initial resting potential (records A and B). This finding indicates that the lower stable potential level (to which the membrane approaches following abolition) undergoes a progressive lowering during activity. At the

end of an unabolished action potential, when the membrane undergoes a natural transition from the upper stable state to the lower, this lower stable state is at the level of a full sized undershoot.

It has been shown in the nodal membrane that there is no absolute refractoriness following an abolished action potential (26). In Fig. 8 an example of the results of a corresponding observation in the squid axon is presented. The records in the upper row show the refractoriness following an unabolished action potential of a TEA-treated axon. As the interval between the two re-

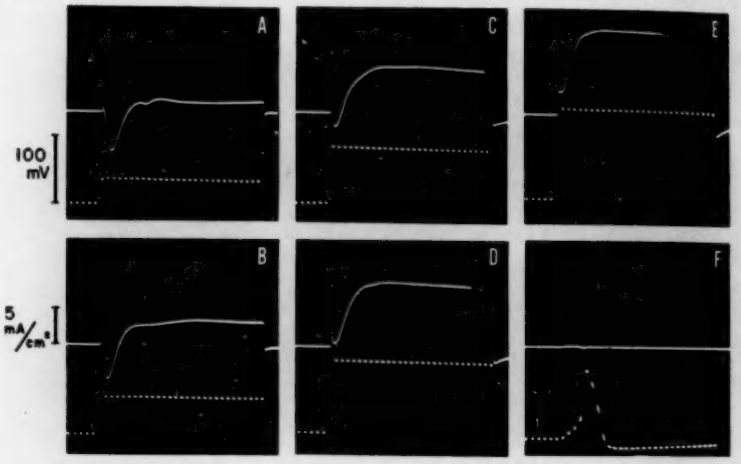

FIG. 9. Records of membrane current associated with rectangular depolarization of a normal giant axon taken with the arrangement of Fig. 2. The dotted lines in records A to E show the time courses of the membrane potential maintained by the method of voltage clamp. Record F was taken by passing a short pulse of outward current through the membrane and later keeping the membrane current at practically zero. Blanking, 0.1 msec. interval. The area of the axon membrane under investigation was approximately 0.11 cm². Temperature 22°C.

sponses decreased, the duration of the second response was greatly reduced. The spike amplitude was also reduced during the refractory period. When the first response was abolished in its early phase, all the signs of the refractoriness following the first response were almost completely wiped out.

4. Voltage-Current Relation.—Using the experimental arrangement shown diagramatically in Fig. 2, it was possible to shift the membrane potential from its resting level quickly to a new constant level and to maintain this new level by an automatic adjustment of the membrane current. In order to compare the behavior of a TEA-treated axon with that of a normal axon, "voltage-clamp" experiments were done on normal as well as on TEA-treated axons. The records in Figs. 9 and 10 show the time courses of the membrane potential

(dotted lines) together with the membrane currents required to maintain these potential levels.

In these records, as well as in the previous ones, an *upward* deflection of the potential trace indicates *positivity* of the axoplasm potential. For the current traces an *upward* deflection corresponds to an *outward* current through the axon membrane. According to this convention, the behavior of a simple ohmic resistor is represented by the parallelism between the deflections of the two traces.

Our results obtained from normal axons, one example of which is presented in Fig. 9, are very similar to those reported by Hodgkin *et al.* (16). The large capacitative current that flows at the onset of a sudden depolarization is barely discernible only as a short break in the current trace. Hodgkin, Huxley, and Katz (p. 426 in reference 16) assume, without presenting supporting evidence, that the capacity of the membrane is connected in parallel with the batteries with series resistances (see Fig. 13A in Discussion) and consequently that the membrane current observed during the voltage clamp is purely ionic. Since we do not consider these authors' argument justifying their separation of this "ionic" current further into sodium and potassium currents as convincing (see Discussion), we describe our experimental results simply in terms of membrane currents and membrane potentials without referring to the physical nature of the potentials and currents.

When the current intensities at the peak of the inward (downward in the figure) surge of the membrane current are plotted against the changes in the membrane potential, it is found that there is a linear relationship between the voltage and the current in a wide range of membrane depolarization (from 50 up to 120 mv. or higher). This is consistent with the results obtained by Hodgkin and Huxley (p. 477 in reference 14). The membrane resistance determined from the slope of this linear relationship was, at 22°C., 7 to 10 ohms·cm.^{2} (3 axons) and, at 11°C., 12 to 14 ohms·cm.^{2} (2 axons). This value of the membrane resistance is considerably smaller than those reported by Hodgkin and Huxley (p. 465 in reference 13) who gave a figure of about 30 ohms for 1 cm.^{2} of membrane.

The membrane potential at which the membrane current at the peak of the inward surge vanishes represents the effective E.M.F. of the membrane at this moment. This value coincides with the membrane potential at the peak of an action potential (compare records D and F in Fig. 9). The final, steady level of the membrane current during voltage clamp varies also linearly as the membrane potential (for membrane depolarization greater than about 40 mv.). This linearity gave a resistance of 9 to 12 ohms·cm^2. and an apparent membrane E.M.F. of 20 to 30 mv. above the resting potential (22°C.). The resistance of the resting membrane measured by clamping the potential at small negative levels was between 2 and 3.5 kilohms·cm.^{2}; the value given by Hodg-

kin *et al.* for the corresponding measurement is 2.3 kilohm·cm.² (p. 440 in reference 16).

The voltage-current relation of the membrane was strongly modified by injection of TEA. Fig. 10 (and also Fig. 12, left), shows the results obtained from axons of which the action potential had been prolonged by TEA. In these records certain features deserve emphasis. The membrane potential at which the peak of the initial surge of current vanishes coincides with the peak of the action potential elicited by a brief stimulating current; undoubtedly this property is common for both the normal and TEA-treated axons. The voltage-

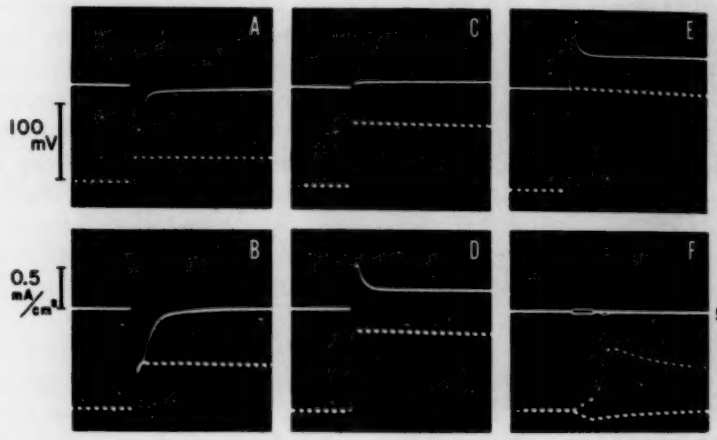

FIG. 10. Records of membrane current associated with rectangular depolarization of the membrane of a squid giant axon treated with tetraethylammonium. The dotted lines show the time courses of the potential difference between the internal and external recording electrodes. Record F shows the action potential of this axon together with the membrane current recorded at a higher recording sensitivity. Blanking, 0.5 msec. apart. The area of the axon membrane under investigation was approximately 0.12 cm². Temperature 22°C.

current relationship at the peak of the initial surge was linear for depolarizations greater than about 50 mv. The membrane resistance determined by this linearity was 25 to 50 ohms·cm.² (6 axons at 22°C.); this finding is consistent with the fact that the maximum impedance loss during activity was smaller in the TEA-treated axons than in the normal.

Another conspicuous feature in the records of Fig. 10 is that the membrane current during the maintained membrane potential is extremely small compared with the case of Fig. 9 for a normal axon. This would not be surprising in view of the fact that the membrane resistance is almost normal during the plateau of an action potential (Fig. 4). When the axon membrane treated with

TEA is clamped at the level of the peak of the action potential as in record C in Fig. 10, the state of the membrane should be very similar to that of a membrane (without a voltage clamp) developing an action potential (record F); the difference would be that, under the conditions of voltage clamp, the membrane potential is maintained at the peak level with the aid of a continuous outward current. The small membrane current during the later phase of voltage clamp is therefore a direct consequence of the relatively high resistance during the plateau of the action potential. (The term "action potential" is used to represent the response of the membrane observed when the membrane current is nearly zero.)

No significant difference was observed between the resting membrane resistance of a TEA-treated axon and that of a normal axon.

5. Stable and Unstable States of the Membrane as Revealed by Voltage Clamp.— When the membrane potential of an axon treated with TEA is clamped at a level of 50 to 75 mv. above the resting potential, the membrane is traversed first by a strong inward current followed by a rapid decrease in the membrane current (Figs. 10B and 11). This amount of sudden depolarization is sufficient to induce a full sized "response" of the membrane as indicated by the appearance of a full sized E.M.F. in the membrane and a full sized reduction in the membrane resistance. Following the peak of this response, the effective membrane–E.M.F. gradually falls and the membrane resistance rapidly increases, resulting in a rapid decrease in the membrane current.

In the experiment of Fig. 11, the membrane potential was suddenly shifted to a second new level at the moment when the membrane current had reached zero at the end of the first step of the maintained membrane potential. This procedure is similar to that used previously by Hodgkin and Huxley (14). When the second voltage step was relatively small, as is the case in the left-hand diagram, the membrane current showed a sudden jump which was roughly proportional to the size of the second step. The membrane resistance estimated from the voltage-current relatior at this moment gave a figure comparable to that of a resting membrane. The procedure of this experiment is evidently analogous to that for measuring the membrane resistance during activity (Fig. 4).

When the second voltage step was large enough to bring the membrane potential to a level slightly below the resting potential (right-hand diagram in Fig. 11), it was found that the membrane again showed a positive (slope) resistance of the same order of magnitude in this range of membrane potential. We call the membrane potential at which the current through the membrane with a positive (slope) resistance vanishes simply the effective membrane–E.M.F.. (In the theory of Hodgkin and Huxley (15), this is considered as a weighted average of the Na and K potentials.) It can be seen in the diagram that there is a stable level of the membrane–E.M.F. at about 10 mv. below the

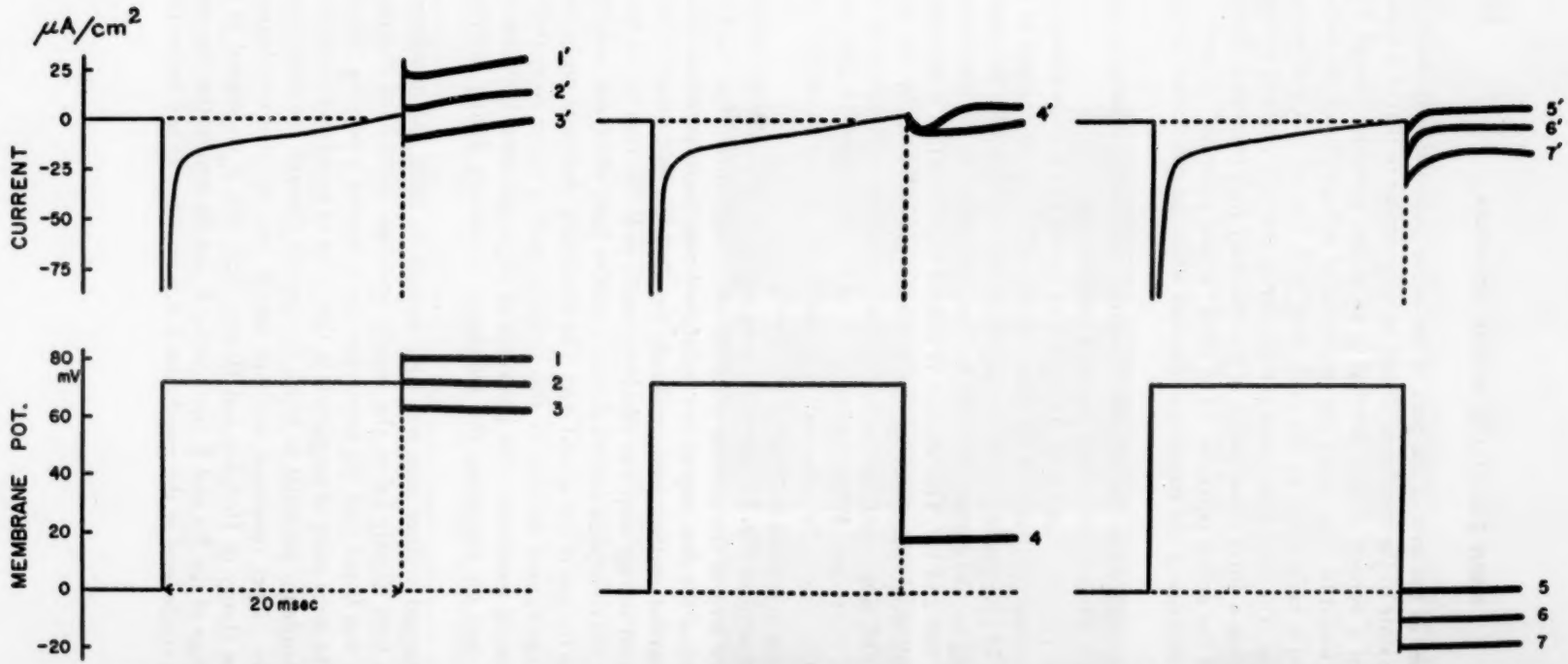

FIG. 11. Diagram showing the time courses of the membrane currents (top) associated with changes in the membrane potential shown at the bottom of the figure. In the two lateral diagrams, traces from three different photographic records were superposed; the corresponding numerals indicate the pairs of traces taken from the same records. In the middle diagram, two records taken at the same membrane potential were superposed. The area of the axon membrane was approximately 0.17 cm². Temperature 22°C.

resting potential; this coincides roughly with the level of the undershoot in the experiment of Fig. 7D. This fact is not surprising since both in Fig. 11, right, and Fig. 7D, the actual experimental procedure is to shift the membrane potential from one level at which the current is zero to another level at which the current is also almost zero.

A state of the membrane described by a positive (slope) resistance and a definite effective membrane–E.M.F. is stable. When the membrane current is reduced to zero, the potential of such a membrane approaches the level of

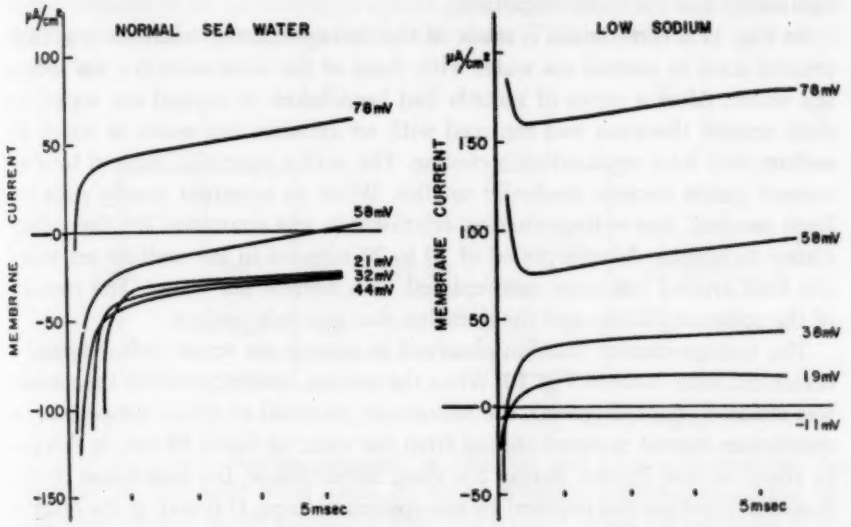

FIG. 12. Effect of replacing normal sea water around the axon with sodium-free sea water upon the voltage-current relation of an axon treated with tetraethylammonium. The sizes of membrane depolarization are given. (It is possible that there were some sodium ions remaining in the medium when the records reproduced in the right-hand diagram were taken.) The area of the axon membrane under investigation was approximately 0.19 cm². Temperature, about 22°C.

the E.M.F. at the rate determined by the product of the membrane resistance and the membrane capacity.

It has been shown (Fig. 7) that, between the upper and lower stable states, there is an "unstable" state at which the membrane current may vanish for a short period of time but the membrane potential is extremely labile. The diagram in the middle of Fig. 11 shows the voltage clamp counterpart of the demonstration of this unstable state. When the second voltage step was at the level (4) in the diagram, the membrane current sometimes stayed at a negative (inward) level for more than 10 msec. and sometimes it shifted rapidly

to a positive level. At this level the behavior of the membrane current was entirely unpredictable under the voltage clamp conditions.

6. Effect of Low Sodium in the Medium.—The first conspicuous effect of replacing the sodium ions in the sea water with choline is a rapid decrease in spike amplitude and duration. This is another demonstration of the similarity between the action potentials of the cardiac muscle and of the squid axon under TEA; Draper and Weidmann (7) and Brady and Woodbury (3) have shown a similar effect of a low sodium medium upon the vertebrate heart. We have not investigated the quantitative relationship between the external sodium concentration and the spike amplitude.

In Fig. 12 a comparison is made of the voltage-current relations in a TEA-treated axon in normal sea water with those of the same axon in a low sodium sea water. After a series of records had been taken in normal sea water, the fluid around the axon was replaced with an artificial sea water in which the sodium ions were replaced with choline. The action potential induced by short current pulses became gradually smaller. When an apparent steady state had been reached, the voltage-current relationship was examined by the voltage clamp technique. After a period of 10 to 20 minutes in low sodium sea water, the fluid around the axon was replaced with normal sea water. The recovery of the spike amplitude and the duration was as a rule perfect.

The voltage-current relation observed in normal sea water (left diagram) is consistent with those in Fig. 10. When the sodium concentration in the medium was reduced (right diagram), the membrane potential at which the peak of the membrane current reversed shifted from the value of about 88 mv. in this case to about 45 mv. Except during the short initial phase, the membrane current flowed in the direction imposed by the applied voltage. It is seen in the diagram that increasingly stronger currents are needed to maintain the membrane potential at higher levels. There was a considerable amount of rectifying action in the membrane treated with TEA; but we received the impression that the rectifying action in such axons was less than that in axons without TEA.

DISCUSSION

Although we know at present nothing about mechanism of prolongation of the spike duration by TEA, the configuration of a prolonged action potential of the squid giant axon is very familiar to many physiologists. The similarity between this prolonged action potential and the normal response of the vertebrate cardiac muscle has been mentioned under Results. The similarity between the response of the crustacean muscle fiber under tetrabutylammonium (8) and the squid response under TEA has also been pointed out in connection with the time course of the membrane resistance during activity. The response of the frog (or toad) motor nerve fiber under such drugs as emetine, brucine, sinomenine, hypertonic NaCl solution (25), strychnine (21), or azide bears

also some resemblance to the prolonged squid response. The toad nerve fiber after prolonged tetanic stimulation develops action potentials strikingly similar to the prolonged squid responses (24). The action potential of a *Nitella* cell treated with guanidine (22) seems also to bear some resemblance to the action potentials described above.

We made an attempt to explain the mechanism of production of prolonged action potentials in the squid in terms of the sodium theory of Hodgkin and Huxley (15). The fact that the response of a squid axon under TEA is strongly reduced in size and duration by the reduction of sodium ion concentration in the medium is an indication of a high sodium requirement in this excitable membrane. In other words, the squid axon under TEA is, unlike the crustacean

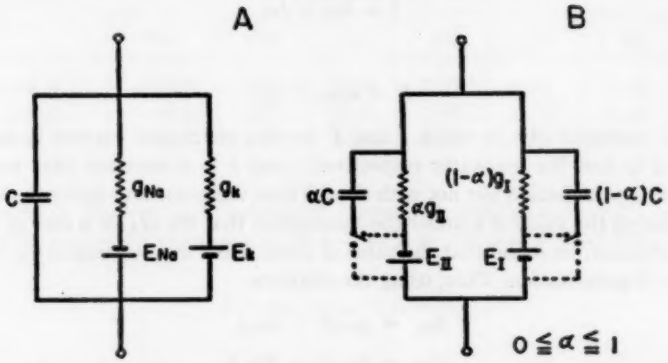

FIG. 13. A, circuit diagram proposed by Hodgkin and Huxley to represent the membrane of the squid giant axon. B, circuit diagram proposed to explain the anomalous behavior of the membrane (Figs. 5D, 6 right, 12 left, etc.) in terms of the "mixed state" of the membrane; the condition $\alpha = 0$ represents one of the two stable states, and $\alpha = 1$ represents the other stable state. Further detail in text.

muscle or *Nitella* cell mentioned above or some of the frog nerve fibers (18), not an exception to the classical form of the sodium hypothesis (Overton, 23). When an attempt was made to interpret the time course of the prolonged action potential in the squid axon in terms of the mathematically formulated, modern sodium theory (*cf.* Fig. 13A), however, a number of difficulties were encountered. We shall discuss some of these difficulties below.

1. If the prolonged action potential is produced by an increase in g_{Na} in the diagram of Fig. 13A, the membrane conductance has to be far higher than its value at rest all during the plateau of the action potential. Actually this is hardly the case. It is not possible to assume a decrease in g_K during activity, since a strong membrane depolarization (up to the level of the plateau) in a medium with low Na content produced marked increase in the membrane conductance (Fig. 12, right).

2. The observation of Fig. 11, middle, and other similar observations strongly

suggest that the time course of the membrane conductance cannot be uniquely determined by the membrane potential. This casts some doubt as to the validity of one of the basic assumptions of the sodium theory (15).

3. When the membrane potential starts to fall at the end of an action potential, there is a decrease in the membrane conductance below the resting level (Fig. 6, right). According to the sodium theory an increase in g_K is expected at this moment. The voltage clamp experiments (Figs. 10 and 11) do not supply any evidence that g_{Na} may start to fall at about this time.

4. In the experiment of Fig. 12, the difference in the membrane currents for the same amount of depolarization in normal and low Na sea water does not reduce to zero during the whole period of maintained depolarization. We tried to separate the membrane currents in this experiment into I_{Na} and I_K by using the equations

$$I = I_{Na} + I_K,$$

and

$$I' = kI_{Na} + I_K,$$

(p. 458 in reference 14), in which I and I' are the membrane current in normal sea water and in low Na sea water respectively, and k is a constant that varies with membrane depolarization but not with time. There was some ambiguity in the process of determining the value of k under the assumption that the $\partial I_k/\partial t$ is zero at the onset of depolarization; we found that the value of about -0.5 was reasonable for the curves for 58 mv. depolarization. Then, using the relations

$$I_{Na} = g_{Na}(V - E_{Na}),$$

$$kI_{Na} = g'_{Na}(V - E'_{Na}),$$

$$I_K = g_K(V - E_K),$$

the values of g_{Na}, g_K and g'_{Na} were calculated. As the constants in these equations we adopted the following figures: $E_K = -10$ mv., $E_l = 10$ mv., $E_{Na} = 90$ mv., and $E'_{Na} = 48$ mv., in which E_l is the membrane potential for the leakage current. The result of calculation is presented in Table I. In this table the value of k in the middle row is the one derived from the actual data. Taking account of a possible error in the determination of k and also trying to show the dependence of the results upon the value of k, the results for three different values of k are given in the table.

The results given in the table impose certain difficulties upon the theory. The values of g'_{Na} marked with asterisks are inconsistent with theory, for a reduction of the sodium in the medium by a factor of one-sixth or less should not increase the sodium conductance of the membrane. The calculated total membrane conductance g is too large to be reconcilable with the data of Fig. 11, left, or with the result of Fig. 4. It is desirable that these points be reinvestigated by other physiologists.

Under Results the experimental facts have been described in terms of the two stable states of the membrane. Each one of these two states is characterized by its effective membrane–E.M.F. and its resistance (or conductance). The term "stable" is used to describe the tendency of the membrane to settle

down in either one of these two states. Using the symbols I and II to distinguish the two states, we denote the effective membrane–E.M.F. and the conductances in these states by E_I, g_I, E_{II}, and g_{II}. When the membrane potential is slightly raised or lowered by a short current pulse from one of these two states, the rate of the potential change in the following stage, dV/dt, is negative when $(V - E_I)$ or $V - E_{II})$ is positive, and *vice versa*; this is the mathematical expression of the stability of the states.

During activity E's and g's undergo continuous relatively slow changes. In the earlier paper (26) these changes were ascribed to the effect of some metabolic product.

TABLE I

	I_{Na}	kI_{Na}	I_K	g_{Na}	g'_{Na}	g_K	g/g_0
$k = -0.3$	−75.7	22.7	67.8	2.37	1.75	1.00	10.7*
−0.5	−65.6	32.8	57.7	2.05	2.52*	0.85	9.3*
−0.8	−54.7	43.7	46.8	1.71	3.36*	0.69	7.8*

Calculation of "sodium" and "potassium" current, I_{Na} and I_k (in μA/cm.^{2}), and sodium and potassium conductances, g_{Na} and g_K (in $m\mho$/cm.^{2}), for the data of Fig. 12 at $t = 20$ msec and $V = 58$ mv. In this case,

$$I = I_{Na} + I_K + I_l = 0,$$

and

$$I' = kI_{Na} + I_K + I_l = 98.4 \text{ A/cm}^2.$$

The total conductance g represents the sum $(g_{Na} + g_K + g_l)$ and g_0 is the observed conductance in the region $O < V < -20$ mv.

Transitions between these two stable states are induced by changes in the membrane potential. If the change in the membrane potential is large and quick, the transition takes place in a very short time (see Fig. 7). If the membrane potential is suddenly brought to an intermediate level between E_I and E_{II}, a variability or an instability of the membrane (associated with threshold excitation or abolition) follows. The interpretation of this threshold phenomenon based on the "two stable state hypothesis" will be as follows:—

When the membrane is in an unstable, intermediate condition, some part of the membrane is in state I and the remaining part is in state II. Under such circumstances, there is a continuous flow of eddy currents between the spots or patches in state I and those in state II, since $E_I \gtrless E_{II}$. Let α denote the fraction of the area in state II in the membrane of 1 cm^2. Then, the portion of $(1\text{-}\alpha)$ of the membrane of a unit area is in state I. The equivalent circuit to represent this "mixed state" will then be as shown by Fig. 13B. The uncertainty in

the positions of the condensers is represented by the dotted lines. At present we do not know whether the membrane capacity is connected in parallel with the membrane–E.M.F. as postulated in the sodium theory of whether it is in series as suggested by Lorente de Nó (p. 445 in reference 19).

In threshold excitation or abolition $dV/dt = 0$ when the net membrane current I is zero. Therefore,

$$I = (1 - \alpha)\, g_{\mathrm{I}}\, (V - E_{\mathrm{I}}) + \alpha g_{\mathrm{II}}\, (V - E_{\mathrm{II}})$$

(which is the sum of the current flowing through the portion of the membrane in state I and the current through the part in state II) is equal to zero. In threshold excitation of a normal axon, the membrane is represented by a mixture of the patches which are in the state of full excitation (state II) and the portion at rest (state I). When the eddy currents caused by these active patches bring the average (or observable) membrane potential, V, up to 10–15 mv., the condition mentioned above (*i.e.* $dV/dt = 0$ and $I = 0$) is attained. Introducing $I = 0$, $E_{\mathrm{I}} = 0$, $V = 10$ mv., $E_{\mathrm{II}} = 110$ mv. and $g_{\mathrm{II}}/g_{\mathrm{I}} = 300$ into the equation above, it is found that α is only about 0.003 or the active patches occupy only about 0.3 per cent of the surface. The increase in the total membrane conductance at this moment is equal approximately to αg_{II}, which should be about 10 per cent of the membrane conductance at rest, g_{I}. Our direct measurement of the change in the membrane impedance showed that this is the right order of magnitude, but more quantitative investigation is required to clarify this point.

Based on the concept of a "mixed state," it is possible to understand the anomalous behavior of the membrane near the end of a response (Figs. 5D, 6 right, 12 left). If one assumes the rapid falling phase of a prolonged action potential to represent a mixed state, the huge value of $\partial V/\partial I$ observed in this stage will be interpreted as indicating a great dependence of α upon V (g's and E's being nearly independent of V).

It is interesting to point out the similarity between the two diagrams in Fig. 13. In discussing initiation and abolition (or natural termination) of an action potential, the mathematical expressions describing the behavior of the membrane potential are very similar in the two cases. Diagram A in Fig. 13 is a special case of diagram B in which both E_{I} and E_{II} are kept constant. The fundamental difference between the sodium theory and the two stable state hypotheses is that in the latter hypothesis E_{Na} in the sodium theory has lost its original physical meaning and is modified during the course of activity to fit the observed data. The problem whether this modification is necessary or not can be settled when we can answer the question whether or not the theory can offer a reasonable explanation for the prolonged action potentials in the squid axon, the frog myelinated nerve fiber, the cardiac muscle, and also in the crustacean muscle fiber.

It is worth mentioning finally that in Lillie's iron wire nerve model a "response" is produced by transition of the iron surface between the active and passive states. In a personal communication, Dr. U. F. Franck told us that the phenomenon of "abolition" of response can be demonstrated in the nerve model and that there is a striking similarity between the results mentioned in this paper and some of his observations on the model. The electrochemical nature of excitation of passive iron wire has been worked out by many recent investigators (1, 9).

SUMMARY

1. Intracellular injection of tetraethylammonium chloride (TEA) into a giant axon of the squid prolongs the duration of the action potential without changing the resting potential (Fig. 3). The prolongation is sometimes 100-fold or more.

2. The action potential of a giant axon treated with TEA has an initial peak followed by a plateau (Fig. 3). The membrane resistance during the plateau is practically normal (Fig. 4). Near the end of the action potential, there is an apparent increase in the membrane resistance (Fig. 5D and Fig. 6, right).

3. The phenomenon of abolition of action potentials was demonstrated in the squid giant axon treated with TEA (Fig. 7). Following an action potential abolished in its early phase, there is no refractoriness (Fig. 8).

4. By the method of voltage clamp, the voltage-current relation was investigated on normal squid axons as well as on axons treated with TEA (Figs. 9 and 10).

5. The presence of stable states of the membrane was demonstrated by clamping the membrane potential with two voltage steps (Fig. 11). Experimental evidence was presented showing that, in an "unstable" state, the membrane conductance is not uniquely determined by the membrane potential.

6. The effect of low sodium water was investigated in the axon treated with TEA (Fig. 12).

7. The similarity between the action potential of a squid axon under TEA and that of the vertebrate cardiac muscle was stressed. The experimental results were interpreted as supporting the view that there are two stable states in the membrane. Initiation and abolition of an action potential were explained as transitions between the two states.

We wish to express our gratitude to Dr. U. F. Franck, Dr. A. M. Shanes, Dr. W. H. Freygang, and Dr. C. S. Spyropoulos for their advice and criticism. We are indebted to Dr. Lorente de Nó for supplying us with a sample of tetraethylammonium chloride solution.

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Note Added to Proof.—Recently del Castillo and Suckling (*Fed. Proc.*, 1957, **16**, 29) also expressed the view that the size of a subthreshold response is determined by the number of active patches or spots in the membrane.

THE CONTRIBUTIONS OF NORMAL AND ANOMALOUS OSMOSIS TO THE OSMOTIC EFFECTS ARISING ACROSS CHARGED MEMBRANES WITH SOLUTIONS OF ELECTROLYTES

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(Received for publication, January 11, 1957)

INTRODUCTION

The term "anomalous osmosis" is commonly used to denote all those osmotic phenomena arising with solutions of electrolytes which seem to be contrary to the common experience that the flow of liquid across a membrane which separates a solution from pure solvent (or a more dilute solution) occurs ordinarily toward the side of the (more concentrated) solution, and at a rate roughly proportional to the concentration difference. In the relatively rare instances of "anomalous negative osmosis" a flow of liquid occurs toward the side of the pure solvent (or the more dilute solution). In the not infrequent instances of "anomalous positive osmosis" the rate of movement of liquid towards the side of the solution depends in an involved manner on the concentration difference across the membrane, the flow rates in a medium range of concentrations being much higher than with more concentrated solutions. Anomalous osmotic effects occur across swelling membranes such as rubber and, more commonly, across non-swelling, porous membranes. This latter effect, anomalous osmosis across porous membranes, the topic treated in this paper, is an electrochemical phenomenon; its magnitude is closely correlated with the product of the electrokinetic charge of the membrane and the dynamic membrane potential which results from the diffusion of electrolyte across the membrane. The details of the mechanism of anomalous osmosis are still controversial and the subject of continuing investigation (1–4).

Anomalous osmosis has been of interest to physiologists since the early reports of Dutrochet because it appears to offer some basis for an explanation of the translocation of liquids across living membranes which cannot be explained on the basis of the concept of normal Pfeffer-van't Hoff osmosis (5–

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10). However, its true role in physiology is still not clear. The published data do not demonstrate clearly that anomalous osmosis can occur to a significant extent under conditions which are at least superficially similar to those most frequently encountered in physiology, particularly in animal physiology. Past experiments have dealt almost exclusively with systems in which a membrane separates a solution of a single electrolyte from distilled water or an extremely dilute solution of the same electrolyte. Also, with rare exceptions, these experiments have been restricted to the measurement of pressure differ-

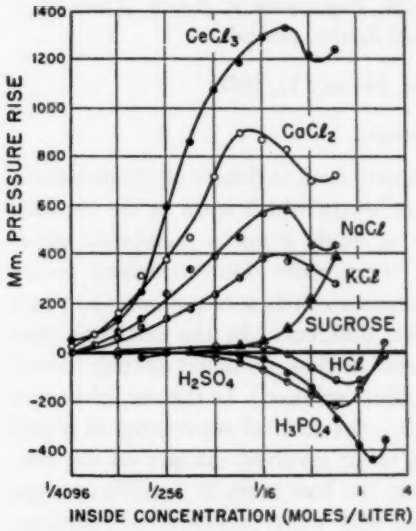

FIG. 1

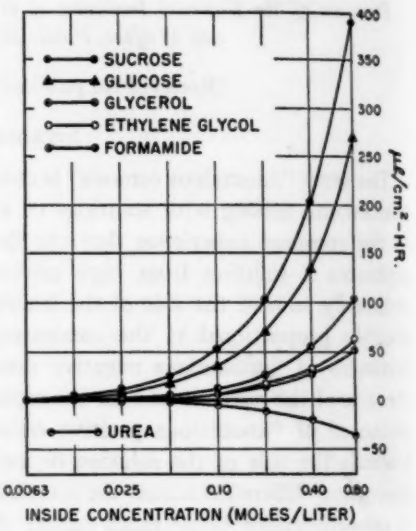

FIG. 2

FIG. 1. Anomalous osmosis across a protamine collodion membrane (data by Abrams and Sollner (12)).

FIG. 2. The rates of movement of liquid across a collodion membrane separating solutions of several non-electrolytes at different concentrations from distilled water.

ences developed across the membrane after an arbitrary time interval (11). While such experiments may be pertinent to certain aspects of plant physiology, information of greater value to animal physiology would be obtained from experiments with systems in which the membrane separates two solutions of different composition, both of which are within the physiological concentration range. Furthermore, information on the rate at which liquid is transported across the membrane against a relatively small pressure would be of greater significance.

A major difficulty in the evaluation of the physiological significance of anomalous osmosis, as well as in its detailed physicochemical study, stems

from the fact that with few exceptions the conditions in systems in which anomalous osmosis arises are such that the simultaneous occurrence of normal osmosis must be presumed. For any detailed investigation of anomalous osmosis, it is therefore necessary to find a method which permits a quantitative distinction between the contributions of normal and anomalous osmosis toward an over-all osmotic effect. The present paper deals with this problem.

Theoretical Considerations

In past studies on anomalous osmosis the contribution of normal osmosis to the over-all osmotic effect arising with a solution of an electrolyte was estimated by comparing the magnitude of this effect with that brought about by a solution of equal concentration of some arbitrarily chosen non-electrolyte. This is illustrated in Fig. 1, in which, following the example of Loeb, sucrose was used as the non-electrolytic solute. The membrane used was an electropositive, preferentially anion-selective protamine collodion membrane (12).

Fig. 1 shows that the osmotic effects with the non-electrolyte sucrose, given by the curve connecting filled triangles, are directly proportional to the concentration of the solute, as would be expected for normal osmosis (13–15), (the curvilinear relation in the figure being a consequence of the semilogarithmic nature of the plot). With the electrolytes, however, no semblance of such proportionality between concentration and osmotic efficacy exists; in several instances anomalous negative osmosis, a transfer of liquid from the side of the solution to the side of the solvent occurs (12).[1] In the past the deviations of the curves found with electrolytes from those found with the non-electrolytic reference substance were assumed to represent the extent of true anomalous osmosis.

Whether or not any arbitrarily chosen non-electrolyte can conceivably be presumed *a priori* to represent a meaningful reference substance for the determination of the contribution of normal osmosis to over-all osmotic effects with electrolytes, is readily decided by comparing the osmotic behavior of different non-electrolytes. Fig. 2 presents the results of some typical osmotic experiments with a collodion membrane of about the same porosity as those used customarily in the study of anomalous osmosis. The experimental data in Fig. 2 are the initial rates of transport of liquid across the membrane, expressed in microliters per square centimeter and hour. (The method used in these experiments is described below.)

[1] The pressure rises with the solutions of neutral salts and of sucrose recorded in Fig. 1 were measured 20 minutes after the test tube–shaped membrane, fitted with a capillary manometer tube and filled with solution, was suspended in a large volume of distilled water. In the experiments with acids, the membrane was filled with water, the acid solution being at the outside; the pressure rises were recorded after 10 minutes.

It is evident from Fig. 2 that the osmotic effects caused by the solutions of different non-electrolytes at the same concentrations differ greatly. Also, it is worthwhile to note that with urea the osmosis is negative; liquid is transported from the side of the solution towards the side of the pure solvent (14, 15). The data prove that it is impossible to find a non-electrolyte which could be used as a universal reference substance for all electrolytes, and likewise that it is impossible to choose, *a priori*, a proper non-electrolytic reference substance for any one specific electrolyte.

A solution of this difficulty lies in the use of each electrolyte as its own reference solute. All existing theories of the mechanism of anomalous osmosis lead to the conclusion that this end could be accomplished by abolishing the electrokinetic charge of the membrane, so that even with electrolytes only normal osmotic effects could occur. The change of the membranes from the charged to the uncharged state, or *vice versa*, must be accomplished without any essential change in the geometrical structure of the membrane; otherwise the osmotic effects observed with the membrane in the two states would not be strictly comparable. This means that the membrane must be non-swelling or must not show any significant change in its state of swelling when its state of charge is altered. From the experimental point of view it would be advantageous if the change in charge could be brought about reversibly.

A satisfactory membrane of this nature is suggested by some of the classical experiments on anomalous osmosis by Loeb (16). With proteinized membranes in contact with electrolyte solutions having a pH equal to the isoelectric point of the protein, the typical N-shaped curves characteristic of anomalous osmosis, such as those shown in Fig. 1, were not obtained. With isoelectric membranes the curves obtained with electrolytes were similar to those obtained with non-electrolytes. Further, proteinized amphoteric membranes, when charged positively or negatively in solutions having pH values significantly different from that of the isoelectric point of the protein used, yielded anomalous osmotic effects like those shown in Fig. 1 for a protamine collodion membrane in the positively charged state.

It is reasonable to assume that with the proper amphoteric, non-swelling membranes the osmotic effect obtained with the membrane in the isoelectric, uncharged[2] state is equal to the contribution of normal osmosis to the over-all osmotic effect obtained with the membrane in the charged state; that is, normal osmotic effects with electrolytes are dependent only upon the geometrical structure of the membrane and are independent of its state of charge. It follows, then, that the contribution of anomalous osmosis towards the over-all osmotic effect with the membrane in a charged state would be the difference

[2] The isoelectric proteinized collodion membrane is not, of course, truly uncharged. It is more accurately described as a balanced mosaic of negative and positive charges.

between the osmotic effects with the membranes in the charged and in the isoelectric state.

The amphoteric material of choice would be one with the isoelectric point near pH 7. With such material, the osmotic effects with the membrane in the isoelectric state can be determined with solutions which require only the most minute additions of acid or alkali to adjust them to the proper pH; more important, the membrane will be positively and negatively charged in solutions containing acid and alkali of such low concentrations that the osmotic effects due to these additives will be insignificant. A material known to be suitable for the preparation of collodion matrix membranes which fulfills very closely the requirement of an isoelectric point near pH 7 is oxyhemoglobin with an isoelectric point of pH 6.75.

EXPERIMENTAL

The purpose of the experimental part of this paper is (*a*) to demonstrate that the contributions of normal and anomalous osmosis toward the over-all osmotic effects which arise with solutions of electrolytes across charged membranes can be determined by the suggested method with the aid of amphoteric membranes; and (*b*) to determine the extent of "true" anomalous osmosis for a variety of electrolytes with positively as well as with negatively charged membranes.

In order to have systems which are better defined and of greater potential physiological significance than those studied in the past, we have measured the osmotic effects arising not between the solution of an electrolyte and pure water but between two solutions of the same electrolyte, the concentrations of which are both of the same order of magnitude, a 2:1 concentration ratio being chosen. These experiments were carried out over a wide range of concentrations including the range of interest in mammalian physiology. The solutions were stirred to maintain the concentration difference across the membrane as nearly as possible identical with that between the bulk of the two solutions, a feature which is missing in nearly all earlier studies.

Preparation of the Membranes.—Collodion membranes of high porosity, of the dialyzing type, were made by a method similar to that used extensively in recent years in this laboratory: A 4 per cent solution of collodion (parlodion Mallinckrodt or Baker collodion cotton, U.S.P.) in a mixture of equal volumes of ethyl ether and absolute ethanol was poured over rubber-coated test tubes (25 × 100 mm.) rotating at a constant speed (18 to 20 R.P.M.) in the horizontal position.[3] The film thus formed was

[3] The immersion in the water shrinks the membranes slightly, making it extremely difficult to remove them intact from the casting tubes. To obviate this difficulty several procedures were attempted: swelling of the membrane in dilute ethanol solutions, prior coating of the casting tubes with starch, powdered talc, caramelized sugar, or rubber. The last procedure was found to be most suitable. A rubber coating is

allowed to dry for several minutes; when thicker membranes were desired, a second layer of collodion solution was applied 3 minutes after the first. Thereafter the tubes were immersed in distilled water and washed for several hours. Finally the skins of rubber covering the test tubes, together with the membranes were slipped off the test tubes and the rubber bags removed from inside the membrane by gentle traction. The membranes were mounted on tightly fitting glass rings of about 20 to 25 mm. length, to which they were secured with linen thread.

The porosity of membranes so prepared depends greatly on the drying time prior to their immersion in water and on the ambient temperature and relative humidity. In general, the porosity is greater the shorter the drying time, the lower the temperature, and the greater the relative humidity. Conditions that yielded one-layer membranes which in the proteinized state produced maximal anomalous osmotic transport rates were for instance: drying time, 4 minutes; temperature, 22°C.; relative humidity, 20 per cent. At the same temperature and a relative humidity of 45 per cent, the drying time had to be lengthened to 6 minutes. Membranes with satisfactory characteristics could not be obtained at relative humidities greater than 50 per cent.

These membranes were proteinized with oxyhemoglobin by the method of Loeb (16). Whole horse blood was centrifuged and the serum decanted. The erythrocytes were three times resuspended in 0.17 M sodium chloride solution, centrifuged, and the supernatant liquid decanted. After the last decantation, distilled water of approximately four times the volume of the erythrocytes was added to hemolyze the cells. Into the oxyhemoglobin solution thus obtained the collodion membranes were immersed for 24 hours. After thorough washing in distilled water, they were ready for use. These membranes could be stored in water to which a crystal of thymol had been added as a preservative for as long as 6 months without undergoing significant changes in their osmotic properties. They were 50 to 100 μ thick; their water content was 60 to 75 volume per cent; and their active area when mounted on the glass rings was about 50 cm^2.

With these membranes, one would expect no significant change in porosity on altering their state of charge. To test this point, the rate of filtration of water was measured with a typical oxyhemoglobin collodion membrane at pH 6.75, 4.0, and 10.0, the membrane being isoelectric, positively charged, and negatively charged, respectively, at these pH values. The rates of filtration were 12.1, 12.5, and 12.4 μl./cm^2.-hr. under a pressure head of 10 cm. of water; this is constant well within the limits of experimental error. Furthermore, osmotic experiments with solutions of non-electro-

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formed on the casting tubes by dipping them in a 60 per cent solution of prevulcanized latex and curing the adhering layer of latex at 60–80°C. for several hours. Though the rubber-covered tubes cannot be washed before the collodion membranes are cast on them, they do not impart to the collodion membranes any material which influences the electrochemical activity of the membranes. Membranes prepared in this manner, produced with electrolytes osmotic effects identical with those obtained with membranes cast over glass test tubes not covered with rubber.

For the latex solution used, prevulcanized natural latex compound, Gl-15-C, we wish to thank the General Latex and Chemical Corporation, Cambridge, Massachusetts.

lytes yielded essentially identical results with such a membrane in the charged and uncharged states. Any differences in the state of swelling of the adsorbed oxyhemoglobin at the different pH values are obviously too small to be of significance in the fairly large pores of the membranes used.

The arrangement for the *measurement of the osmotic effects* consisted of a rubber stopper (fitting the glass rings of the membranes) which carried a short calibrated glass tube of about 8 mm. diameter, a narrow bore glass tube with a stopcock, and two magnetic stirrers, small alnico permanent magnets covered with lucite and mounted on glass axles held by a strip of plastic. For the experiments, the rubber stopper was inserted firmly into the ring of the membrane which had been filled with solution, and the position of the meniscus in the glass tube adjusted by means of the stopcock, to a level just above the stopper. The volume of solution in the assembled apparatus was about 35 ml. This assembly was then immersed in a beaker containing the "outside"

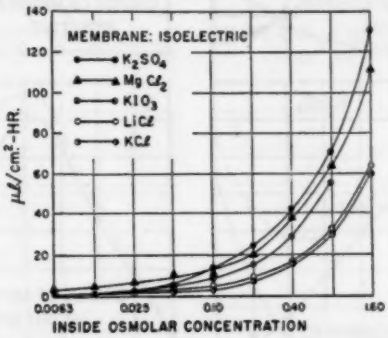

FIG. 3. Rates of movement of liquid across an oxyhemoglobin membrane in the isoelectric state separating solutions of various electrolytes with concentration ratios of 2:1. (The indicated concentrations refer to the more concentrated solutions.)

solution, (350 ml. in the case of solutions and 1000 ml. in the case of pure water), up to a level identical with that of the meniscus in the calibrated tube. The inside solution was stirred by rotating the magnets with a regularly interrupted magnetic field supplied by a large, externally mounted electromagnet. The outside solution was stirred vigorously by a motor-driven propeller.

A 5 minute period was allowed to elapse before the volume change of the inside solution was read; the liquid transport rates in microliters per square centimeter and hour were calculated from such readings. In 5 minutes, the concentration difference across the membrane in most instances decreased by about 5 to 10 per cent. Thus, the calculated liquid transport rates are on the average less than 5 per cent lower than initial rates at zero time. The use of shorter experimental periods would have decreased this discrepancy, but would have increased the error of measurement in the case of systems producing small volume changes. As routine, each measurement was repeated with fresh solutions until succeeding results agreed within 2 μl./cm.^{2}-hr. The changes in hydrostatic pressure which accompanied the changes in volume of the

inside solution were in all instances too small to cause a significant error due to filtration.

All experiments were carried out at room temperature which remained virtually constant during any given experiment and did not vary to a significant degree during any series of experiments with a given solute.

For the experiments with the membrane in the isoelectric state the solutions were adjusted to a pH of 6.75 by the addition of traces of HNO_3 or KOH as required. The

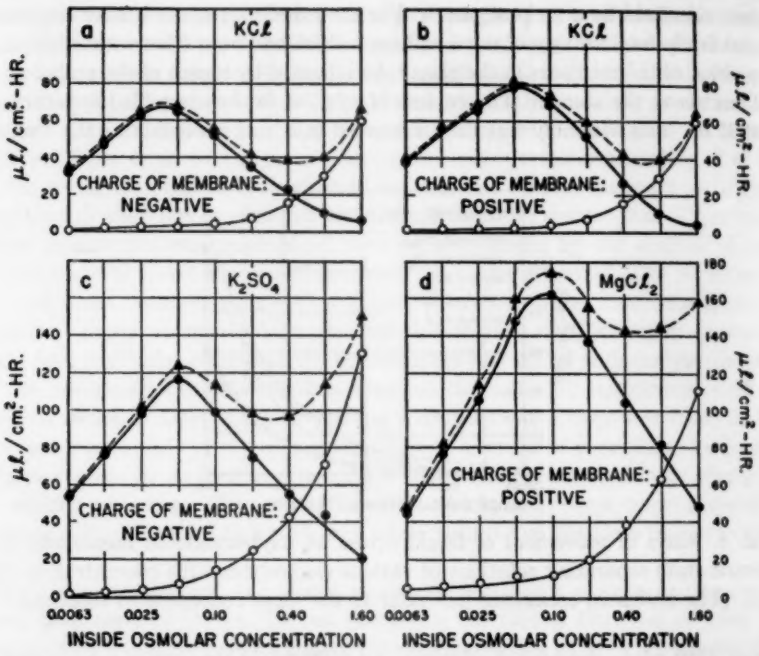

FIG. 4. Rates of movement of liquid across an oxyhemoglobin membrane in the isoelectric and in a charged state separating solutions of an electrolyte with a concentration ratio of 2:1. Four instances in which anomalous osmosis is positive at all concentrations. —○—, membrane in the isoelectric state; —▲—, membrane in a charged state; —●—, true anomalous osmosis.

solutions for the experiments with the membrane in the positively charged and the negatively charged states were similarly adjusted to a pH of 4.0 and 10.0.

The representative experiments reported below were all performed with the same oxyhemoglobin membrane (having a filtration rate of 10 μl./cm.²-hr. at 10 cm. water pressure) and thus are strictly comparable. The electrolytes used were chosen to furnish examples of both positive and negative anomalous osmosis, with the membrane in both the positively and the negatively charged

state (1–3, 12). The concentration of the inside solution was in all the experiments twice that of the outside solution.

Fig. 3 shows the results of some experiments with the membrane in the isoelectric state. The concentrations of the electrolytes are expressed as osmolarities, defined here as the products of the molar concentrations and the number of ions formed on complete dissociation. (Accordingly, osmolarity and molarity of non-electrolytes are numerically identical.)

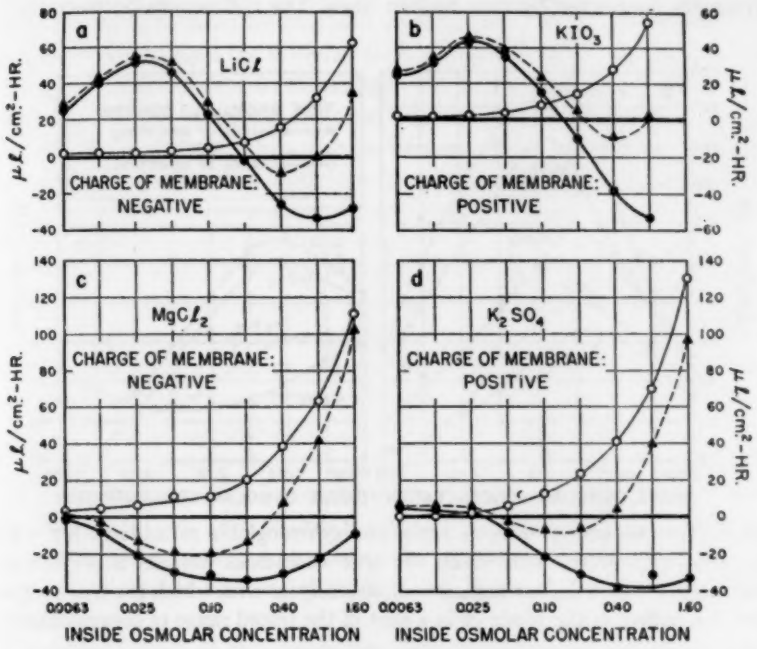

FIG. 5. Rates of movement of liquid across an oxyhemoglobin membrane in the isoelectric and in a charged state separating solutions of an electrolyte with a concentration ratio of 2:1. Four instances in which anomalous osmosis is negative in the whole or in a part of the tested range of concentrations. —○—, membrane in the isoelectric state; —▲—, membrane in a charged state; —●—, true anomalous osmosis.

The curves of Fig. 3 show that the osmotic effects with an isolectric membrane and a given electrolyte are proportional to the concentration difference across the membrane, as are the osmotic effects with non-electrolytes shown in Fig. 2 (the apparent curvilinear relations in Figs. 2 and 3 being a consequence of the conventional semilogarithmic plot). This demonstrates that with isoelectric membranes electrolytes behave osmotically in a "normal" manner, indistinguishable from non-electrolytes.

Fig. 4 (*a–d*) gives the osmotic effects obtained with four systems in which the anomalous osmosis that arises is positive throughout the whole concentration range tested. Fig. 5 (*a–d*) presents four systems in which the anomalous osmosis is negative either over the whole or a part of the tested range of concentration.

The data with the membrane in the isoelectric state are represented by unfilled circles connected by thin solid lines; those obtained with the membrane in a charged state, positively or negatively as the case may be, are represented by triangles connected by thin broken lines. The differences between the two,

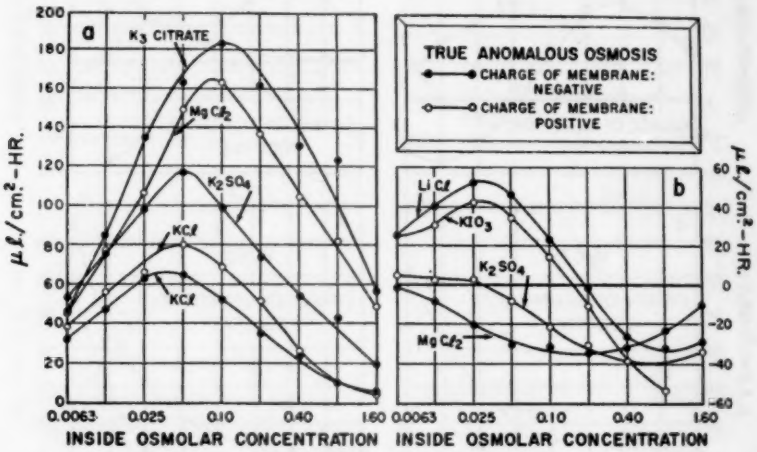

FIG. 6. True anomalous osmosis across an oxyhemoglobin membrane. Fig. 6 *a*, five instances of electrolytes with which the true anomalous osmosis is positive at all concentrations; Fig. 6 *b*, four instances of electrolytes with which the true anomalous osmosis is negative in the whole or in a part of the tested range of concentrations.

the "true" anomalous osmoses, are indicated by filled circles connected by heavy solid lines. In Figs. 4 and 5 the osmolar concentrations of the inside solutions which are the more concentrated, are given on the abscissa, and the rates of transport of liquid in microliters per square centimeter and hour on the ordinate. Negative values indicate a net movement of liquid into the outside, more dilute solution.

To facilitate the comparison of the behavior of the different electrolytes, the data on true anomalous osmotic transport rates are assembled in Fig. 6. Fig. 6 *a* presents the data on the systems which showed only true positive anomalous osmosis (with the results of a series of experiments with potassium citrate added); 6 *b*, the data on the systems which showed negative anomalous osmosis at least at some concentrations.

DISCUSSION

From the physicochemical point of view, the data presented do not require extensive comment. Those in Fig. 3 on the osmotic behavior of several electrolytes with an isoelectric membrane show a linear relation between osmotic effect and concentration difference for each electrolyte, and, with regard to the differences in the osmotic behavior of the various electrolytes at the same concentration, a direct relation between osmotic effect and ionic size. These regularities are good evidence that with isoelectric membranes, normal osmotic effects alone are produced by solutions of electrolytes. Since the porosity of the membrane is unchanged by bringing it to a charged state, the osmotic effects obtained with the isoelectric membrane can be assumed to be equal to the normal osmotic components of the total osmotic effects obtained with the membrane in a charged state. Thus, the use of amphoteric membranes seems to be a valid and practicable way to determine meaningful reference values for estimating the extent of true anomalous osmosis in systems in which normal and anomalous osmoses occur simultaneously.

The shape and relative heights of the curves representing true anomalous osmosis in Figs. 4 and 5 demonstrate that the results presented here are in agreement with those of earlier more qualitative studies. True positive anomalous osmosis is greatest in a medium concentration range and is greater with electrolytes having multivalent ions of the same sign of charge as the membrane than with electrolytes having univalent ions of the same charge (and the same counter ions). The maximal rates obtained were 80 μl./cm.^{2}-hr. with 0.05 osmolar KCl and 165 μl./cm.^{2}-hr. with 0.1 osmolar $MgCl_2$ and the membrane in the positive state, and 185 μl./cm.^{2}-hr. with 0.1 osmolar K_3-citrate and the membrane in the negative state. Maximal true negative anomalous osmosis occurs at higher concentrations and is smaller, 30 to 55 μl./cm.^{2}-hr. at concentrations of 0.2 to 0.8 osmolar.

The main purpose of this paper has thus been achieved; namely to demonstrate a method which allows the determination of the extent of true anomalous osmosis, and to present a selection of quantitative data on this effect.

The described method for the determination of the normal and of the anomalous osmotic components of the over-all osmotic effects arising with solutions of electrolytes may, with caution, be applied also to those non-amphoteric membranes which can be discharged to a sufficient extent by the addition of low concentrations of some electrolyte other than that to be tested for its osmotic efficacy. This should be possible with low-charge density membranes of weak acid or weak base character, as is borne out by some of Loeb's experiments with collodion membranes of, as we know now, low-charge density in their pore system (17, 18). Such membranes were discharged adequately at pH 3.0. High charge density membranes, particularly those of strong acid and strong base character such as the now widely used polyelectrolyte-impregnated collodion

matrix and ion exchanger membranes, cannot be discharged by concentrations of acids or bases which would be compatible with the purpose at hand (19, 20). The discharge of such membranes with polyvalent ions, while feasible, may introduce a variety of complications. Here the extent of anomalous osmosis with any given electrolyte may be estimated best by reference to an amphoteric membrane of the same structural character, which shows a similar osmotic behavior with non-electrolytes and also, when in the charged state, with the electrolyte under consideration.

From the physiological point of view it is of considerable significance that fairly large true anomalous osmotic effects occur in the range of concentrations of mammalian body fluids. In the past, several prominent investigators have doubted that anomalous osmosis, as an electrokinetic effect, can occur at concentrations above 0.05 M, and thus were inclined to the conclusion that osmotic effects arising at higher concentrations must be due solely to normal osmosis (10, 21–23). The data presented here demonstrate definitely that the general electrochemical conditions at the physiological concentration level are such that anomalous osmosis does occur to a considerable extent with suitable proteinized membranes, a result that might have been anticipated on the basis of the known high electrophoretic mobilities of proteins in solutions of fairly high ionic strength.

It also might be worth noting that at concentration levels below those occurring in the mammalian organism, anomalous osmosis is an even more effective mechanism, a fact which might be of particular interest in plant physiology.

The experiments reported here are not as pertinent to mammalian physiology as would be desirable since they were carried out with systems in which the membrane separated two solutions of different concentration of the *same* electrolyte. For the physiologist it would be more valuable to know whether or not anomalous osmotic effects of significant magnitude arise if a membrane separates two solutions of different composition but of the same osmolar concentration. This question will be taken up in a subsequent paper.

SUMMARY

The osmotic effect arising across a porous membrane separating the solution of an electrolyte from water (or a more dilute solution) is ordinarily due to both normal osmosis, as it occurs also with non-electrolytes, and to "anomalous" osmosis. It is shown that the normal osmotic component cannot be measured quantitatively by the conventional comparison with a non-electrolytic reference solute. Anomalous osmosis does not occur with electroneutral membranes. Accordingly, with membranes which can be charged and discharged reversibly (without changes in geometrical structure), such as many proteinized membranes, the osmotic effects caused by an electrolyte can be measured both when only normal osmosis arises (with the membrane in the electroneutral

state) and when normal as well as anomalous osmosis occurs (with the membrane in a charged state). The difference between these two effects is the true anomalous osmosis. Data are presented on the osmotic effects across an oxyhemoglobin membrane in the uncharged state at pH 6.75 and in two charged states, positive at pH 4.0 and negative at pH 10.0, with solutions of a variety of electrolytes using a concentration ratio of 2:1 over a wide range of concentrations. The rates of the movement of liquid across the membrane against an inconsequentially small hydrostatic head are recorded instead of, as conventional, the physiologically less significant pressure rises after a standard time.

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THE METAMORPHOSIS OF VISUAL SYSTEMS IN THE SEA LAMPREY

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(Received for publication, February 7, 1957)

The lampreys are members of the most primitive living group of vertebrates, the cyclostomes, last survivors of the ancient class Agnatha, the jawless vertebrates. Having evolved in directions peculiarly their own, they have also preserved many primitive characters which other vertebrates have lost. This is as true biochemically as it is anatomically (*cf.* Florkin, 1949). For example, lamprey hemoglobin is unique among vertebrates, possessing properties found otherwise only among invertebrates (*cf.* Wald and Riggs, 1951–52).

Some years ago I examined the visual system of the sea lamprey, *Petromyzon marinus* (Wald, 1941–42 *b*). The retinas were found to contain both vitamins A_2 and A_1 in the approximate proportion 9:1. The pigment epithelium and choroid layers of the eye also contained a predominance of vitamin A_2, about 4 times as much as A_1. The livers, however, contained vitamin A_1 alone. I made no attempt at that time to extract visual pigment from the retina; but on the strength of the great preponderance of vitamin A_2, and Walls's report (1935) that dark-adapted retinas of this species are "truly purple" in color, I concluded that this retina contains porphyropsin. This lent some support to the view that porphyropsin rather than rhodopsin may have been the primitive vertebrate visual pigment.

Recently Crescitelli (1955–56) has extracted the visual pigment from retinas of *P. marinus*, and found it to be rhodopsin. This occurrence of rhodopsin in a vertebrate which spawns in fresh water—indeed, Crescitelli's animals had never left fresh water—seems at variance with the observation that such animals in general possess predominantly porphyropsin (Wald, 1945–46; 1952). Crescitelli noted also that the occurrence of rhodopsin in the lamprey favors the view that rhodopsin rather than porphyropsin was the primitive vertebrate visual pigment.

On reexamination it has come out that both Crescitelli's observations and

* This investigation was supported in part by funds from The Rockefeller Foundation and the Office of Naval Research. I am greatly indebted to Dr. Ruth Hubbard for help with the experiments.

mine were correct. The examples of the sea lamprey with which Crescitelli worked were newly metamorphosed animals, on their journey downstream which on the sea coast would have led them to the ocean, and in the case of his animals would have led them into Lake Huron. The animals with which I had worked were sexually mature adults, on their migration upstream from the ocean. The downstream migrants have, as Crescitelli found, the rhodopsin system alone. The mature upstream migrants, however, possess the porphyropsin system virtually alone. The sea lamprey exhibits a biochemical metamorphosis of visual systems, comparable with that previously observed among amphibia (Wald, 1945–46; 1952). The significance of this metamorphosis in terms of the lamprey's life cycle and phylogeny will be discussed below.

Material

Adult, sexually mature lampreys were taken from the Oyster River in Durham, New Hampshire, on the night of May 22, 1956.[1] These animals had collected below a dam on their spawning migration upstream from the ocean. Twenty-four of them were brought alive to a laboratory at the University of New Hampshire, and the eyes were taken out at once under red light. The retinas (46 in all) were dissected out and frozen immediately on dry ice. The remaining eye tissues—sclera, choroid, and pigment epithelium—were frozen separately. The dissection had been performed in frog-Ringer solution, and this also was saved.

One of the retinas, looked at in white light, appeared almost colorless. It has been noted earlier that the retina of the adult sea lamprey contains very little visual pigment (Wald, 1941–42). With the idea that perhaps considerable numbers of outer segments might have broken off the retina into the Ringer solution, or might have remained embedded in the pigment epithelium, we saved all these materials for extraction.

Back at the Harvard Laboratory, the retinas were ground by mortar and pestle in 40 per cent sucrose dissolved in phosphate buffer, pH 7. The suspension, in a small plastic centrifuge tube. was layered over with phosphate buffer, and centrifuged for 10 minutes at 40,000 R.P.M. in the Spinco preparative ultracentrifuge. On such treatment, the rod outer segments, in large part fragmented, collect as a thin layer in the interface between the sucrose solution and buffer. The entire supernatant, containing the suspended rods, was poured off, and the sediment was reground in 40 per cent sucrose. In all, three such flotations were performed. On pouring off the supernatants, the sucrose was diluted by mixing with buffer solution. As a result, on recentrifuging the mixed supernatants, all the rod outer segments sedimented. They were washed once in distilled water, and then were left to harden in 4 per cent alum solution for 35 minutes. They were collected by centrifuging, washed again in distilled water, and in phosphate buffer of pH 6.9, and finally were stirred into 0.55 ml. of 2 per cent digitonin solution and left for 1¾ hours in the refrigerator

[1] I am grateful to Professors Paul A. Holle and Edwin Scheier of the University of New Hampshire for help with procuring and handling these animals; and to my coworkers, Ruth Hubbard, Norman Krinsky, and Timothy Goldsmith, with whose help this expedition was brought to a successful outcome.

to extract the visual pigment. This suspension was centrifuged for 20 minutes at 25,000 R.P.M., and the clear extract of visual pigment poured off.

The Ringer washings were centrifuged, and the sedimented material mixed with the remaining eye tissues. This material was ground thoroughly, and treated exactly as described above.

We then had to wait for 7 months, until Dr. Vernon C. Applegate, who had obtained animals also for Crescitelli, could send us downstream migrants.[2] Fourteen such animals were sent us from Rogers City, Michigan, on the shore of Lake Huron, by Air Express on December 5, 1956. They were dark-adapted overnight in running tap water and the eyes removed under red light. After cutting away the cornea and lens, the whole fundus of each eye was frozen. We also dissected out and froze the livers of all these animals. The whole fundi of twenty-seven eyes were ground with mortar and pestle and suspended in 40 per cent sucrose. They were treated exactly as has already been described, the rod outer segments being floated out in three successive grindings and centrifugings, washed with distilled water, hardened in alum solution, washed again with distilled water and neutral phosphate buffer, and finally stirred into 0.5 ml. of 2 per cent digitonin solution to extract in the cold for 2 hours.

Observations

The absorption spectrum of the visual pigment of the downstream migrants is shown in curve *a* of Fig. 1. It is a typical rhodopsin spectrum, displaying an α-band with λ_{max} 500 mμ, and an unusually prominent β-band with λ_{max} about 340 mμ. Between these maxima lies a typical minimum at 400 mμ.

This pigment was bleached by exposure to the concentrated light of a 160 watt microscope lamp passing through a yellow Corning *3482* filter, which transmits only wavelengths longer than 555 mμ, and so fails to isomerize the retinene which is formed. The first such exposure, for 3 minutes, yielded curve *b*. This was followed by a second, 5 minute exposure, which yielded curve *c*. Curve *c* possesses λ_{max} about 373 mμ, typical of the bleached all-*trans* retinene$_1$–protein complex at the pH at which these measurements were made (7.5); and a slightly higher maximal extinction than rhodopsin itself, also typical at this pH. Apparently lamprey rhodopsin possesses very nearly the same molar extinction as cattle rhodopsin (*i.e.*, about 40,000 per retinene equivalent (Wald and Brown, 1953–54).

A small granule of potassium borohydride was stirred into this solution. The spectrum immediately afterward had changed to *d*. The borohydride had reduced the retinene formed by bleaching to vitamin A, with λ_{max} about 328 mμ, the correct position for all-*trans* vitamin A_1 in digitonin solution. There is no suggestion of the presence of vitamin A_2, which possesses an absorption maximum at about 355 mμ.

To the solution which yielded curve *d*, methanol was added to a concentration of 60 per cent, and the mixture was extracted with petroleum ether. The petroleum ether extract was transferred to 0.5 ml. of chloroform. To this

[2] I am greatly indebted to Dr. Applegate, Director of the Hammond Bay Fishery Laboratory in Rogers City, Michigan, for providing these animals.

in an absorption cell in place in the spectrophotometer, 2 ml. of saturated antimony chloride solution in chloroform was added, and the absorption spectrum of the resulting blue product recorded at once. This is shown in Fig. 3. It exhibits the single absorption band maximal at about 618 mμ typical of vitamin A_1 in this test.

These observations therefore confirm and extend those of Crescitelli. The downstream migrant sea lamprey possesses in its retina the rhodopsin system, based upon retinene$_1$ and vitamin A_1 alone.

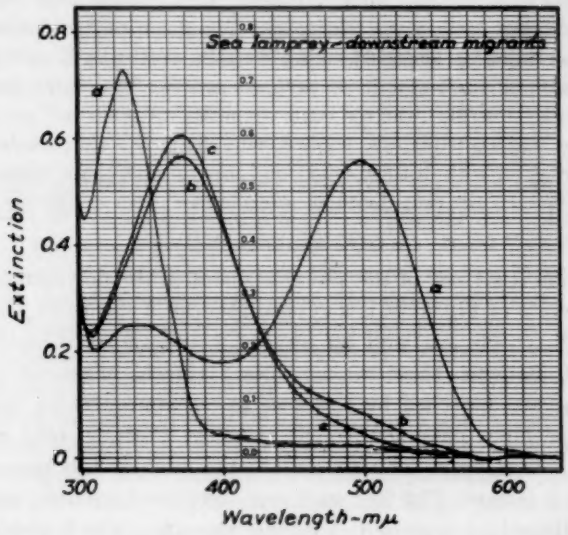

FIG. 1. The visual pigment of the downstream migrant sea lamprey, and the products of its bleaching. (*a*) Absorption spectrum of the visual pigment, a typical rhodopsin, with α-band at 500 mμ and β-band at 340 mμ. After exposure for 3 minutes to a yellow (non-isomerizing) light, the spectrum has changed to (*b*); and following a second, 5 minute exposure to the same light, to (*c*). This is the spectrum of all-*trans* retinene with opsin. On stirring with potassium borohydride, the retinene is reduced to all-*trans* vitamin A (*d*). pH 7.5.

Comparable data from the mature upstream migrants are shown in Fig. 2. The visual pigment (curve *a*) has its absorption maximum at about 518 mμ. The porphyropsins of fresh water fishes lie characteristically at about 522 ± 2 mμ. The visual pigment of the upstream migrant, therefore, lies just lower in wavelength than a typical porphyropsin, either because of a small intrinsic difference, or a very small admixture of rhodopsin (see below).[3]

[3] We have obtained a porphyropsin with λ_{max} 517 mμ synthetically, by incubating the hindered *cis*-isomer neo-b retinene$_2$ with cattle opsin (Wald, Brown, and Brown, unpublished experiments).

This pigment was bleached by exposure to the concentrated light of a 160 watt microscope lamp passing through Corning filter *2412*, which passes only wavelengths longer than about 610 mμ. After 19 minutes' exposure, the spectrum had changed to curve *b*. The solution was exposed 2 minutes longer through a Jena *OG 2* filter, which transmits only wavelengths longer than 550 mμ. This yielded curve *c*. It possesses the maximal absorption at about 401 mμ characteristic of all-*trans* retinene$_2$ in digitonin solution at this pH (7.5).

A droplet of potassium borohydride solution was now stirred into this solu-

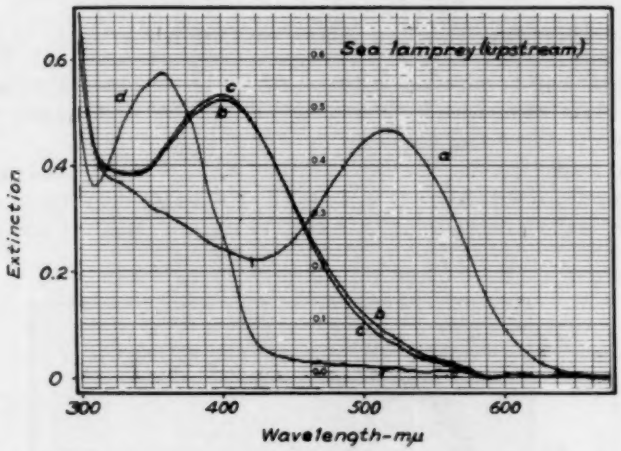

FIG. 2. The visual pigment of the upstream migrant sea lamprey, and the products of its bleaching. (*a*) Absorption spectrum of the visual pigment, a porphyropsin with λ_{max} 518 mμ. On exposure to orange light for 19 minutes, the spectrum has changed to (*b*); and after 2 minutes' further exposure to yellow light, to (*c*). This is the spectrum of all-*trans* retinene$_2$ and opsin. Stirring with potassium borohydride reduces the retinene$_2$ to vitamin A_2 (*d*). pH 7.5.

tion. The spectrum changed to curve *d*. This possesses a maximum at about 357 mμ, characteristic of all-*trans* vitamin A_2 in digitonin solution.

The solution which yielded curve *d* was diluted with methyl alcohol to a concentration of 60 per cent, and extracted with petroleum ether. The petroleum ether extract was transferred to 0.5 ml. chloroform. To this in place in the spectrophotometer about 2 ml. of antimony chloride reagent was added, and the spectrum was recorded immediately. This is shown in Fig. 4. It exhibits predominantly the absorption maximum at about 690 mμ characteristic of vitamin A_2. There is in addition a small inflection in the neighborhood of 615 to 620 mμ, caused by the presence of a small amount of vitamin A_1.

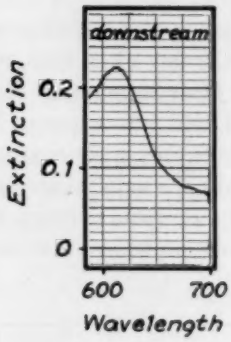

FIG. 3

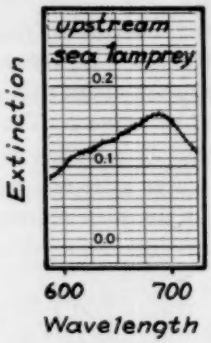

FIG. 4

FIG. 3. Antimony chloride test with an extract of the bleached visual pigment from downstream migrant sea lampreys (Fig. 1 *d*). The test displays only the absorption band maximal at 618 mμ characteristic of vitamin A_1.

FIG. 4. Antimony chloride test with an extract of the bleached visual pigment from upstream migrant sea lampreys (Fig. 2 *d*). The test shows the presence of vitamin A_2 (λ_{max} 690 mμ), accompanied by a trace of vitamin A_1 (λ_{max} 615 mμ).

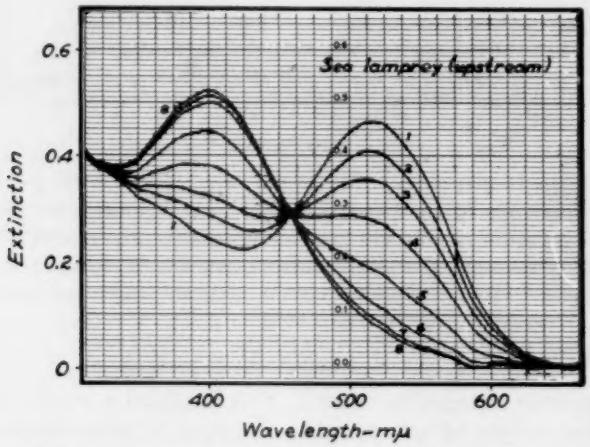

FIG. 5. Test for homogeneity of the visual pigment of upstream migrants. The pigment (1) was exposed repeatedly to a non-isomerizing orange light, containing only wavelengths longer than 610 mμ, and the spectrum recorded (2) after 15 seconds' exposure, (3) after a total of 30 seconds, (4) 1 minute, (5) 2 minutes, (6) 4 minutes, (7) 9 minutes, and (8) a total of 19 minutes' exposure. Such a deep orange light should have bleached porphyropsin much faster than rhodopsin, if both pigments were present. The sharp isosbestic point at 457 mμ is good evidence that a single pigment (porphyropsin) is bleaching.

The proportions are much as we have found before in the retina of the sexually mature lamprey.

Do the upstream migrants possess porphyropsin alone, or mixed with a very small amount of rhodopsin? We attempted to test the visual pigment for homogeneity by bleaching it by stages in the orange light passing through a Corning *2412* filter. Since this transmits only wavelengths longer than about 610 mμ, it should bleach porphyropsin much more rapidly than rhodopsin.

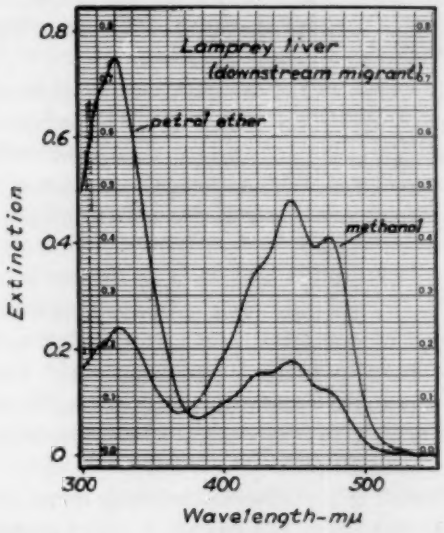

FIG. 6. Carotenoids of the liver of the downstream migrant sea lamprey. The absorption spectra show the presence of vitamin A_1 (λ_{max} 325 mμ) and a xanthophyll (λ_{max} 423, 448, and 476 mμ). The spectra show the fractions obtained by partitioning the extract between petroleum ether and 90 per cent methyl alcohol. The xanthophyll enters primarily the methanol layer, showing it to be mainly unesterified; whereas the vitamin A enters primarily the petroleum ether, showing it to be present mainly as esters.

One might expect, therefore, that the porphyropsin would bleach first, and that the spectra in the later stages of bleaching would shift more and more toward those typical of rhodopsin. This test is shown in Fig. 5. Its most striking result is that bleaching under these conditions exhibits every sign of complete homogeneity. The best evidence for this is the sharp common or isosbestic point at about 457 mμ, showing that one is dealing with the transformation of a single molecular species into another. From these data one would infer that the upstream migrants possess porphyropsin alone, with little or no admixture of rhodopsin.

It appears from our measurements that the concentration of visual pigment is considerably greater in the retinas of downstream than in upstream migrants. In both we find the extinction of visual pigment per eye nearly the same: 0.012 when the total visual pigment from one retina is dissolved in 1 ml., and measured in a 1 cm. layer. Since, however, the adult retina has about 4 times the area of that of a downstream migrant, the *concentration* of visual pigment in the adults is only about one-fourth of that in downstream migrants. Indeed I have not otherwise seen a retina well supplied with rods that contains so little visual pigment as the upstream migrant sea lamprey; and Kühne (1878) has much the same thing to say of the retinas of (presumably mature) *Lampetra fluviatilis*.

In my earlier paper (1941–42) I described observations on the liver vitamin A in the larval lamprey and in the migrating adult. In both cases only vitamin A_1 was found. Crescitelli (1955–56) also has shown the antimony chloride test of an extract of the livers of downstream migrants, which again is characteristic of vitamin A_1. Apparently the liver of the sea lamprey contains vitamin A_1 alone throughout its life history.

Fig. 6 shows direct absorption spectra of an extract of the livers of downstream migrants. About 1 gm., fresh weight, of livers taken from 14 animals was ground with anhydrous sodium sulfate to a dry powder, and was extracted by gently stirring into several portions of petroleum ether. The extract was deep yellow in color. It contained vitamin A_1 (λ_{max} 325 mμ) and a xanthophyll or hydroxy-carotenoid with λ_{max} (423), 448, and 476 mμ (leaf xanthophyll: 420, 448, 478 mμ in petroleum ether).

Fig. 6 shows the result of partitioning the whole extract between petroleum ether and 90 per cent methanol. The vitamin A enters primarily the petroleum ether layer (epiphasic), showing it to exist in the liver primarily as esters; whereas the xanthophyll enters primarily the methanol layer (hypophasic), showing it to be present mainly as the free alcohol. Lamprey xanthophyll is more strongly hypophasic than leaf xanthophyll, and has not yet been finally identified. One gm. of fresh liver contains about 80 μg. of xanthophyll and about 125 μg. of vitamin A.

DISCUSSION

In the course of its life cycle the sea lamprey undergoes two metamorphoses. It begins as an ammocoete larva, living buried in the sand or mud of its natal stream; and blind, its rudimentary eyes buried in the tissues of the head. After 4 to 5 years in the larval condition, it metamorphoses. This involves a fundamental anatomical reconstruction, which prepares the animal for a predatory life in which it is parasitic upon fish, and which includes the appearance of well formed eyes. This metamorphosis is completed with the animal still living as did the larva, though no longer feeding. Then the lamprey mi-

grates downstream to the ocean or a lake for the growth phase, which lasts 1½ to 3½ years.

At the end of this phase the sea lamprey undergoes a second metamorphosis to the sexually mature adult. This is not usually spoken of as a metamorphosis, yet it has that essential character. In the growth phase the sexes can hardly be told apart without a dissection; even the gonads look alike. Now the sexes differentiate visibly. The gonads mature, and special external structures are formed for depositing eggs and sperm. The males develop a ropelike ridge along the back. At this time also either sex or both may assume golden mating tints. I have noticed that the livers of the mature animals are bright green, though those of larvae and downstream migrants are the usual reddish brown. These changes are completed in the environment of the growth phase, whether lake or sea. Then the animals migrate upstream to spawn. They have stopped feeding, and indeed may be unable to digest and absorb food. They have before them only the act of reproduction; shortly afterward both sexes die.[4]

The lampreys with which we have been concerned were examined on their migrations downstream and upstream, having shortly before completed the associated metamorphoses. It is clear that the alterations which constitute metamorphosis include profound changes in the chemistry of visual excitation.[5]

Rhodopsin and the use of vitamin A_1 in vision are characteristic of marine and land vertebrates, whereas porphyropsin and vitamin A_2 are characteristic of fresh water forms (Wald, 1938–39; 1945–46; 1952). The downstream migrant lamprey, which under ordinary conditions is about to enter the ocean, appears to anticipate this change biochemically by assuming, while still in fresh water, the marine type of visual system. Conversely the upstream migrants, even toward the beginning of their migration, already have the fresh water form of visual system. That is, the biochemical changes in the eye anticipate the migrations. In this they mimic the anatomical changes of metamorphosis. Indeed with regard to more adaptive aspects of metamorphosis, such anticipation of the environment to come is essential, for the animal cannot move to the new environment until metamorphosis has made it ready.

How do these observations reflect upon the phylogeny of vertebrate vision? There is reasonable support for the view that the vertebrate stock originated in fresh water (Smith, 1932; Romer and Grove, 1935). Coupled with the observation that porphyropsin is characteristically associated in vertebrates with fresh water existence—even with temporary phases of fresh water ex-

[4] For information concerning the life history and metamorphoses of the lamprey I am indebted principally to the work of Gage (1927) and Young (1950).

[5] I have tacitly assumed in this discussion that the lamprey retains the rhodopsin of the downstream migrants throughout the growth phase, changing to porphyropsin only as it approaches sexual maturity. The precise time of this change is still to be determined.

istence—this site of origin raises the possibility that porphyropsin rather than rhodopsin was the primitive pigment of vertebrate vision. The discovery of porphyropsin in lampreys some years ago seemed to support this possibility. The present observation that in the sea lamprey the visual system metamorphoses between rhodopsin and porphyropsin does not alter this position materially. The significant point is that in these most primitive of living vertebrates porphyropsin occupies much the same position as in fishes and amphibia. As yet it has not been found at all among higher vertebrates.

I have discussed elsewhere biological parallelisms between euryhaline fishes and amphibia (1945–46; 1952). Euryhaline fishes occupy much the same position between fresh water and marine fishes that amphibia occupy between fresh water fishes and land vertebrates. Metamorphoses and migrations are characteristic of both groups. All the euryhaline fishes I have examined were in their growth phase, in middle life. All of them possessed either mixtures of the rhodopsin and porphyropsin systems, in which the type associated with the spawning environment predominated, or that type alone. On the other hand, amphibia, examined at various phases in their life cycles, exhibit distinct metamorphoses of visual systems. Thus the bullfrog tadpole enters metamorphosis with the porphyropsin system, and emerges from it with the rhodopsin system. This is the type of performance we find in the sea lamprey. It represents a distinct variation from the relatively stable patterns found in euryhaline fishes, while still preserving the general association of porphyropsin with fresh-water life, and rhodopsin with marine and terrestrial habit.[6]

The change from porphyropsin to rhodopsin in the metamorphosis of the bullfrog has the appearance of a biochemical recapitulation. It seems to recall in the act of metamorphosis the change from porphyropsin in an ancestral fresh water fish to the rhodopsin of land-living forms. How then interpret the reverse change from rhodopsin to porphyropsin in the sea lamprey? Does it imply that rhodopsin preceded porphyropsin in lamprey evolution?

I think the answer to this question lies in the recognition that this change in

[6] As the visual system of the bullfrog changes from porphyropsin to rhodopsin when it metamorphoses from a tadpole, its hemoglobin also changes profoundly (McCutcheon, 1936; Riggs, 1951–52). It seemed worthwhile to look for comparable changes in the lamprey. The properties of lamprey hemoglobin had already been examined in upstream migrants (Wald and Riggs, 1951–52). Dr. Riggs has now also examined the hemoglobin of downstream migrants, obtained through the kindness of Dr. Vernon Applegate from the same source and at the same time as our animals (personal communication). He has found no differences from our earlier measurements in the shape of the oxygen equilibrium curve, the affinity for oxygen, or the change in affinity with pH (the Bohr effect). Apparently the downstream and upstream migrants possess the same hemoglobin. This leaves open the possibility that the larvae have a different hemoglobin; *i.e.*, that there may be a change in hemoglobin associated with the primary metamorphosis.

the sea lamprey is part of the *second metamorphosis*. I have found precisely the same phenomenon in the New England spotted newt, *Triturus viridescens* (Wald, 1946; 1952). Following a larval period in fresh water, this animal undergoes a first metamorphosis to the terrestrial red eft. Later it undergoes a second metamorphosis to the sexually mature adult, which returns to fresh water to spawn. I found that the red eft contains predominantly rhodopsin, the water phase adults predominantly porphyropsin. The *second metamorphosis* involves the transfer from rhodopsin to porphyropsin, just as in the sea lamprey.

The second metamorphosis in an amphibian or migratory fish or lamprey is not to be viewed as the recapitulation of some earlier evolutionary transition, but as the adaptation to a life cycle that involves two environments. To complete the cycle it is necessary to bring the adult back into the original environment to spawn; and this in some instances involves changes which in part reverse those of the first metamorphosis.

It should be interesting now to explore the visual systems of other types of lamprey. All lampreys begin their lives in fresh water; some species never leave this environment. Kühne (1878) reported finding the retinas of *Lampetra fluviatilis*, an anadromous form like *P. marinus*, very feebly purple in color, inclining in hue toward violet or bluish. Presumably this was a sexually mature adult, and apparently its retina contained porphyropsin. Crescitelli cites Lovern *et al.* (1939) as having observed that the eyes of *Lampetra fluviatilis* contain a great preponderance of vitamin A_1 over A_2. These animals were apparently sexually mature adults of average weight 36.6 gm. The authors, however, state only that in the antimony chloride test with an extract of the eyes they could measure a very small extinction (0.015) at 617 mμ and none at 693 mμ. They make no mention of observing a specific absorption band, and lacking this one cannot be sure that any vitamin A was present in the extracts.[7]

On the other hand, Crescitelli reports measurements with a single extract of five sexually mature Pacific coast lampreys (*Entosphenus tridentatus*, an anadromous form) which appeared to contain rhodopsin. In this, *Entosphenus* may be like the bullfrog, which goes over to rhodopsin at its first metamorphosis, and so far as we know retains it thereafter. That is, *Petromyzon marinus* and *Entosphenus tridentatus* among lampreys appear to exhibit the same relations of visual system to life cycle as do the spotted newt and bullfrog among amphibia.[8]

[7] Steven (1950) has measured the spectral sensitivity of the photoreceptors in the tail of the ammocoete larva of the brook lamprey, *Lampetra planeri*, a permanently fresh water form. He found the sensitivity to be maximal at 520 to 530 mμ, and suggests that this may be associated with the presence of porphyropsin. He has measured also the light responses of another cyclostome, the marine hag, *Myxine glutinosa*, a blind animal whose skin photoreceptors appear to be maximally sensitive at 500 to 520 mμ (1955).

[8] Still another variable may enter this situation. Lamprey retinas possess two types

In discussing his discovery of rhodopsin in the sea lamprey, Crescitelli remarks that it would be logical to suppose this to be the primitive vertebrate visual pigment, since all the invertebrate eyes so far examined contain components of the rhodopsin system (vitamin A_1, retinene$_1$; Wald, 1941; 1945–46). Hence porphyropsin as the primitive vertebrate pigment would represent a gratuitous discontinuity in the otherwise smooth transition from invertebrate to vertebrate rhodopsins.

This argument involves a common confusion of contemporary animals with ancestral forms, which I once shared, and have discussed elsewhere (Wald, 1952). Vertebrate eyes were not derived from invertebrate eyes. The only invertebrate phyla which have developed proper image-forming eyes—the Arthropods and Molluscs—were probably not in the line of vertebrate ancestry, nor are their eyes in any way homologous with vertebrate eyes. Each of these phyla developed eyes in complete independence. The Echinoderms, from which we suppose the vertebrates to have sprung, have never developed eyes. No discontinuity is implied therefore in the notion that when vertebrates did develop eyes, they began with porphyropsin.

The alternative view is that, having started with rhodopsin, the vertebrates developed porphyropsin secondarily, in close genetic association with fresh water habit, and particularly with the habit of spawning in fresh water. This was my original thought (1938–39). I think the other view—that porphyropsin was the primitive vertebrate pigment—accords better with the belief that vertebrates originated in fresh water, and with the appearance of porphyropsin among the lampreys.

In any case it is now clear that lampreys toy with the distribution of rhodopsin and porphyropsin in close association with environmental habit, much as do the fishes and amphibia. I say "toy" because it is difficult to attach any special advantage to the use of either pigment in any of the environments in question. The genetic characters which decide the environment seem also to determine the choice of visual system as a gratuitous by-product. Why, particularly if this distribution is so trivial, it follows the same complex patterns in cyclostomes, fishes, and amphibia, is an extraordinarily interesting problem. It may possibly mean that an identical hereditary mechanism—perhaps an identical array of genes—is at work throughout this great span of organisms.

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of visual cell, long and short, which have variously been described as rods or cones. Walls (1935) defends with his usual clarity and passion the view that the short cells are rods, the long ones cones. Among a wide sample of lampreys, he found *Entosphenus tridentatus* to have the highest proportion of short to long cells (8:1), whereas in *P. marinus* this ratio was 3:1, and in the *Lampetras* about 1:1. It should be interesting to know whether these ratios change during the life cycle.

SUMMARY

The life cycle of the sea lamprey, *Petromyzon marinus*, includes two metamorphoses. At the end of a period spent as a blind larva, buried in the mud of streams, a first metamorphosis prepares it to migrate downstream to the sea or a lake for its growth phase. Then, following a second metamorphosis, it migrates upstream as a sexually mature adult to spawn and die.

The downstream migrants have a visual system based upon rhodopsin and vitamin A_1, whereas that of the upstream migrants is based upon porphyropsin and vitamin A_2. The livers contain vitamin A_1 at all stages.

The sea lamprey therefore exhibits a metamorphosis of visual systems, like those observed earlier among amphibia. The presence of porphyropsin in this member of the most primitive living group of vertebrates, as in fishes and amphibia, supports the notion that porphyropsin may have been the primitive vertebrate visual pigment. Its association with fresh water existence throughout this range of organisms also is consistent with the view that the vertebrate stock originated in fresh water. The observation that in the life cycle of the lamprey rhodopsin precedes porphyropsin is not at variance with the idea that porphyropsin is the more primitive pigment, since this change is part of the *second metamorphosis*, marking the return to the original environment. The observation that in lampreys, fishes, and amphibia, porphyropsin maintains the same general association with fresh water, and rhodopsin with marine and terrestrial habit, suggests that a single genetic mechanism may govern this association throughout this wide span of organisms.

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THE ACTIVE TRANSPORT OF PHOSPHATE INTO THE YEAST CELL*

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(Received for publication, January 31, 1957)

The exchanges of phosphate between cellular and intracellular compartments have been studied in a number of cell types. In each case the entry of phosphate into the cell seems to be associated with metabolic processes at the cell surface (1). For example, in red blood cells, the uptake of P^{32}-labelled phosphate is to a large measure dependent on glycolysis (2). It has a high temperature coefficient (3) and is blocked by metabolic inhibitors (2). Studies of precursor relationships and also of phosphate turnover in red cell stroma suggest that phosphate enters the cell by incorporation in the membrane into glycolytic intermediates such as 2,3-diphosphoglycerate and ATP (4, 5). In muscle, precursor relationships with P^{32} also suggest that phosphate enters the cell *via* some phosphorylation reaction (6). However, the latter studies involve the technical difficulty of distinguishing extra- and intracellular orthophosphate in the muscle mass. Autoradiographic studies (7) lend support to the phosphorylation concept. In sea urchin eggs formation of ATP at the cell surface seems to be an integral part of the mechanism of phosphate entry (8).

In microorganisms a dependence of phosphate entry on metabolic reactions is well established (9). Yeast has been most intensively studied. In growing yeast, the uptake of P^{32}-labelled phosphate requires the presence of sugar and is blocked at low temperature (10), and by metabolic inhibitors (9). The phosphate which is absorbed is largely converted to polymerized forms of phosphate (11, 12). The total amount of uptake is dependent on the presence of cations such as potassium and magnesium which are also absorbed (13). It is not clear whether the effect of potassium is due to a stimulation of the entry process for phosphate, or to a maintenance of cation-anion balance within the cell, or to stimulation of metaphosphate formation within the cell.

In the present study some of the properties of the process of phosphate entry into yeast cells have been investigated, including the kinetics of the reaction, the nature of the substrate, the nature of the entering ion, the effect of potassium, and certain other related phenomena.

* This paper is based on work performed under contract with the U. S. Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, New York.

Methods

Non-growing populations of *Saccharomyces cerevisiae* were used. Bakers' yeast (Standard Brands, Inc.) was washed three times with distilled water and then aerated and starved for 18 to 24 hours. Cells were centrifuged again, resuspended in distilled water, and their concentration measured turbidometrically. In order to avoid known effects of inorganic cations, trishydroxyaminomethane, (THAM) or triethylamine (TEA) were used in a buffer mixture with succinic acid (14). In the pH range of 6.5–7.5 in which the buffer is ineffective, the suspensions were continuously titrated with TEA or with HCl.

The suspensions were bubbled with either nitrogen or air for 30 minutes with substrate in order to establish a uniform rate of metabolism. Orthophosphoric acid was then added, and the time of this addition was considered zero for all subsequent measurement. At specified times aliquots were withdrawn from the suspensions, centrifuged quickly, and the decanted supernatant was analyzed for phosphate. In cases in which cells were to be analyzed, they were washed at least twice before analyses were begun. Extraction of soluble cellular phosphate was carried out with cold 10 per cent trichloracetic acid. Specific procedures used in analysis were as follows:

Phosphate—method of Fiske and Subbarow (15) modified by the addition of 15 per cent ethyl alcohol before color development.

P^{32} activity—Geiger counter.

O_2 consumption and CO_2 production—standard Warburg procedure.

Glucose—method of Nelson (16).

RESULTS

In the absence of exogenous substrate, there is only a very slow exchange of cellular and extracellular phosphate (10), an observation confirmed in the present studies. Nevertheless, the extracellular phosphate was found to distribute into a cellular space representing about 10 per cent of the total cell volume, presumably the "cell wall space" demonstrated by Conway (17). This space is permeable to sugars, sodium chloride, and amino acids, but not to peptone or insulin.

Little binding or adsorption of phosphate by the cell surface was observed, even at very low concentrations of phosphate (1×10^{-6} M).

Beyond the initial distribution phenomena, no redistributions of phosphate take place between the resting cell and the medium. However, when glucose is added, there is a rapid uptake of orthophosphate by the cells as measured by its disappearance from the medium (Fig. 1). Furthermore, the rate of disappearance of phosphate is the same when it is measured chemically or by P^{32} activity. Thus the specific activity of the phosphate remaining in the medium does not change even after a large proportion of the phosphate has been absorbed, indicating the absence of exchange of cellular and extracellular phosphate. It must be concluded that the flow of the phosphate is essentially unidirectional, from the medium to the cell.

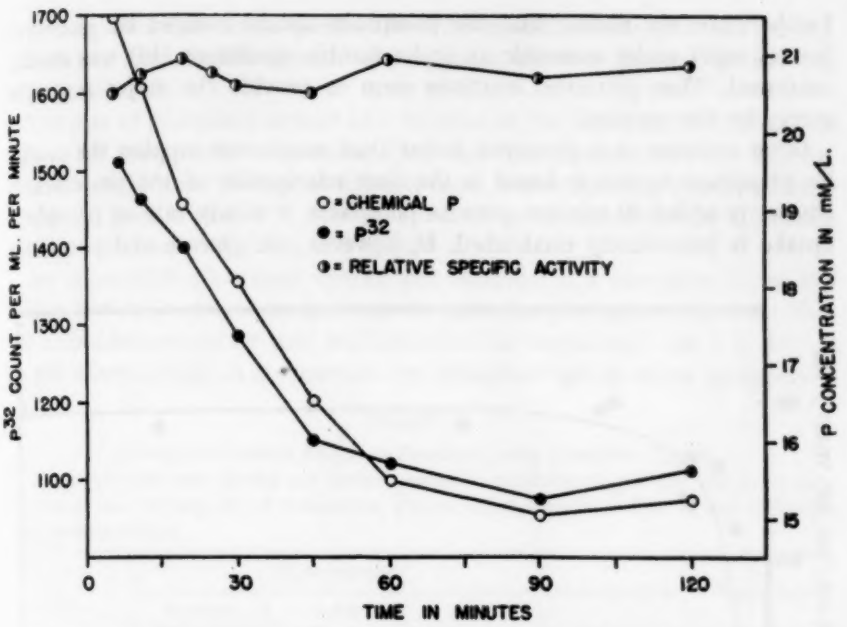

FIG. 1. The disappearance of P^{32} and of chemical phosphate from the medium during the fermentation of glucose by yeast. The yeast concentration was 50 mg./ml. of suspension, pH, 7.0; glucose, 0.2 M; and initial phosphate, 0.022 M.

TABLE I

The Dependence of Phosphate Uptake on Specific Substrate

Experiments were carried out aerobically, and pH was always in the range of 4.5–5.0. Initial concentration: yeast, 100 mg./ml.; substrate, 0.3 M; KCl, 0.02 M; H_3PO_4, 0.01 to 0.02 M.

Substrate	No. of experiments	Rate of phosphate uptake in mM of P/kg. yeast
Glucose	5	254.8
Fructose	4	201.6
Mannose	3	161.4
Lactate	2	10.3
Pyruvate	5	9.1
Acetate	4	0
Ethyl alcohol	4	49.5

Not every substrate will support a rapid uptake of phosphate. In fact, the reaction was relatively specific for the fermentable sugar, glucose, fructose, and mannose (Table I). The respiration of alcohol supported a rate of phosphate uptake only 20 per cent of that found with glucose and the respiration of lactate, pyruvate, and acetate could support only a very low rate of uptake.

Furthermore, the finding that the phosphate uptake induced by glucose is just as rapid under anaerobic as under aerobic conditions (18) was readily confirmed. Thus glycolysis reactions seem to provide the major source of energy for the reaction.[1]

Other evidence that glycolysis rather than respiration supplies the energy for phosphate uptake is found in the time relationship of the processes. If glucose is added 30 minutes prior to phosphate, a steady rate of phosphate uptake is immediately established. If, however, the glucose and phosphate

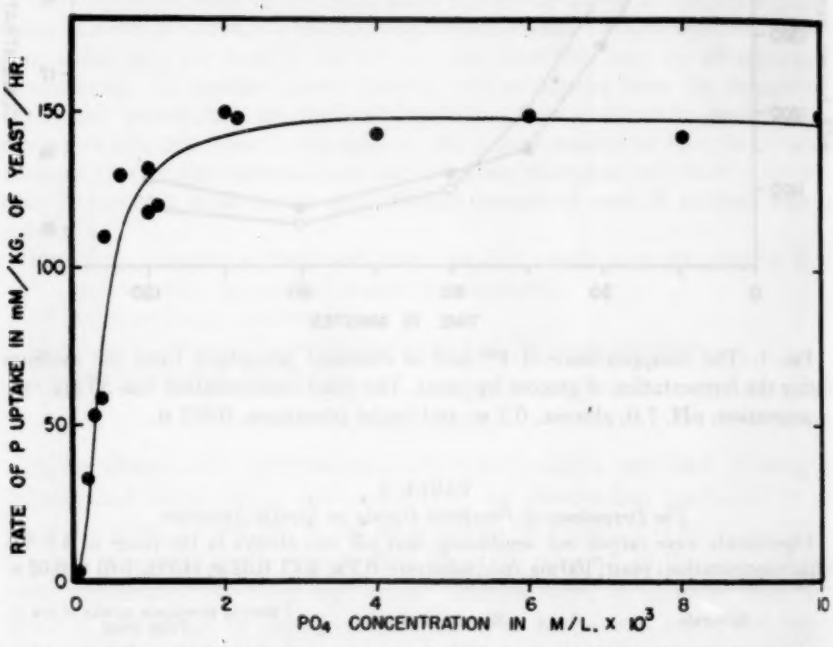

FIG. 2. The relationship between the initial rate of phosphate uptake and the initial phosphate concentration. The yeast concentrations varied from 10 to 50 mg./ml., the pH was 4.0; glucose, 0.2 M; and K^+, 0.02 M.

are added simultaneously, there is a 10 to 15 minute lag period before a steady rate of phosphate uptake is achieved. A similar lag period in the attainment of the maximal rate of fermentation can be demonstrated, whereas no such lag period occurs in the case of respiration.

Although phosphate uptake is dependent on the fermentation of glucose, no apparent stoichiometric relationship exists between the two processes. The maximal rate of phosphate uptake observed was 260 mM per kg. of cells

[1] In this strain of yeast there is a high rate of aerobic glycolysis. Thus even in the aerobic experiment, about 40 per cent of the glucose was fermented.

per hour. The sugar uptake under the same conditions was 1500 mM per kg. of cells per hour. In the absence of phosphate, the rate of sugar uptake was slightly reduced.

The rate of phosphate uptake as a function of the phosphate concentration follows an asymptotic relationship (Fig. 2). The maximal rate for this experiment of 160 mM per kg. of cells per hour, was attained at phosphate concentration greater than 2×10^{-3} M. The data can be fitted by the Michaelis-Menten equation with a Km of 4×10^{-4} M.

An appreciable phosphate uptake was observed at a concentration as low as 2×10^{-4} M. In view of the fact that the orthophosphate concentration within the cells (determined by cold trichloracetic acid extraction) was 2×10^{-2} M or 100 times as high, it is apparent that phosphate can be taken up against a

TABLE II

Changes in Cellular Phosphate Fractions during Phosphate Uptake

The experiment was carried out under anaerobic conditions at pH 4.5. The yeast concentration was 250 mg./ml. of suspension, glucose 0.3 M, phosphate 0.05 M, and potassium when present 0.02 M.

Time	TCA-extractable						Non-extractable		Total cellular	
	Inorganic		Labile		Stable					
	No K	K	No K	K	No K	K	No K	K	No K	K
min.										
0	19.7	26.7	3.8	3.4	17.5	12.1	61.1	71.5	115.1	128.8
10	25.0	36.9	5.8	9.2	22.2	13.8	64.9	86.4	131.7	164.6
20	25.0	25.0	8.0	6.9	25.9	23.5	72.3	119.4	146.6	200.3
40	25.8	26.9	12.2	10.3	21.8	15.9	86.4	125.5	164.6	205.2
60	25.8	25.3	12.9	11.2	24.4	14.9	78.3	108.7	158.1	183.3

Values are in mM of P/kg. of yeast.

large concentration gradient. Furthermore, the gradient was not altered by the influx of phosphate, for the cellular orthophosphate concentration remained constant during the course of the experiment (Table II), the phosphate taken up being entirely channelled into phosphate compounds.

The effects of potassium and of extracellular pH on phosphate uptake are of some interest. Previous studies (15) have demonstrated a marked stimulation of phosphate uptake by potassium. The extent of the potassium effect was found to be dependent on the pH of the medium (Fig. 3). For example, in the range of pH 7–9, there was little K-effect, whereas in the range of pH 3–6, stimulation was observed with a maximum of 700 per cent at pH 4.0. In consequence, the pH optimum for phosphate uptake was shifted in the presence of potassium from pH 6.5 to pH 4.8.

The effect of potassium is a specific one, for sodium was unable to stimulate the phosphate uptake.

The right hand legs of the pH curves of Fig. 3, lie in a position similar to that for the second dissociation of phosphoric acid, suggesting that the monovalent anion, $H_2PO_4^-$ is selectively absorbed in preference to the bivalent anion, HPO_4^{--}.

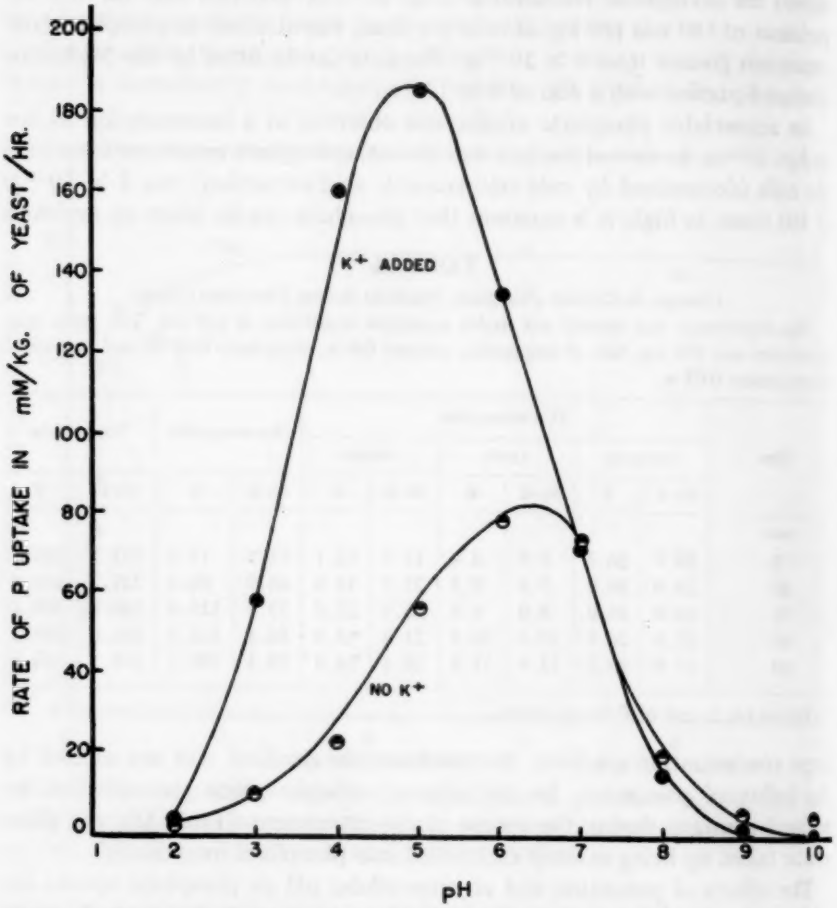

FIG. 3. The effect of pH on the uptake of phosphate in the presence and absence of K^+. The yeast concentration was 50 mg./ml.; the glucose, 0.2 M; and the K^+, 0.02 M.

DISCUSSION

Data have been presented indicating that the entry of phosphate into the yeast cell involves an active transport system:

(*a*) Orthophosphate is taken up against a concentration gradient as large as 100 to 1.

(*b*) The uptake is dependent on specific substrates.

(*c*) The relationship between phosphate concentration and rate of phosphate uptake is asymptotic, suggesting that entry of phosphate involves its combination with a limited quantity of a cellular constituent.

(*d*) During the uptake of phosphate, there is almost no efflux of the same ion, indicating that transport take place across a permeability barrier with a high resistance to phosphate diffusion.

The specific dependence of phosphate uptake on the fermentation of sugars, particularly glucose, is of interest. Of the fermentation reactions in the Emden-Meyerhof scheme, the only one that might be implicated in the esterification of orthophosphate is the glyceraldehyde-dehydrogenase step.

$$\text{Glyceraldehyde-3-PO}_4 + \text{PO}_4 \sim \text{1,3-diphosphoglycerate}$$

The same reaction is strongly implicated in phosphate uptake by red blood cells, on the basis of precursor relationship (5). The phosphate compound which shows the highest P^{32} specific activity early in the course of phosphate uptake is 2,3-diphospholgycerate, a compound in enzymic equilibrium with the 1,3-diphosphoglycerate in the red cell.

Another possibility is phosphorylation by the reverse reaction of phosphatases (20). However, in yeast, the phosphatases of the cell surface can be completely blocked by molybdate, with no effect on phosphate uptake (21).

If a phosphorylation reaction is indeed involved in phosphate transport, this reaction must be located at or near the surface of the cell where contact with extracellular phosphate is possible. Furthermore the reaction must be isolated from the interior of the cell by a permeability barrier to phosphate which prevents exchanges of intra- and extracellular phosphate. Evidence has been presented elsewhere (22) that such is the case, that the glycolytic reactions in the yeast cell are located in a compartment in the periphery of the cell. Phosphorylation at the cell surface *per se* does not explain the absence of phosphate exchanges, for the reactions are themselves reversible and a phosphorylation reaction in equilibrium with both extra- and intracellular phosphate would allow exchanges to occur. It may be presumed, therefore, that an essentially irreversible phosphate transport step across a membrane is involved, in association with the phosphorylation reaction.

The effects of potassium and pH on phosphate uptake are complex. The pH is due in part to the selective uptake of $H_2PO_4^-$ in preference to HPO_4^{--}. In addition the rate of fermentation is influenced by the extracellular pH. For example, in the absence of potassium there are two pH optima, at 4.5 and 8.2 (23). However, neither of these corresponds to the optimum for phosphate uptake at pH 6.5. In the presence of potassium, the fermentation is independent of pH over a broad range (pH 2.5–8.5 for the potassium concentration in the present experiment). Again there is no apparent relation-

ship to phosphate uptake. Thus, the pH effect on phosphate uptake seems to reflect in large a direct action of H^+ on the phosphate transport mechanism.

The stimulation of phosphate uptake by potassium may involve three phenomena:

(*a*) A direct stimulating effect of potassium on the transport mechanism.

(*b*) A shift of the pH in the cell surface region by a membrane-hydrolysis phenomenon. The outer membrane of the cell is impermeable to anions, but permeable to K^+ and H^+. Therefore an increase in the extracellular concentration of K^+ results in alkalinization of the cell surface compartment by an exchange reaction (22). This mechanism accounts for the reversal of the inhibitory effects of H^+ on fermentation by K^+ (14), and perhaps also for the shift in the optima of the pH curves for phosphate uptake (Fig. 3).

(*c*) A balancing of cellular cations and anions. Small quantities of $H_2PO_4^-$ can be taken up without cation absorption at the expense of cellular buffer capacity (cell more acid, medium more alkaline). However, the uptake of phosphate in large quantities for an extended period can continue only if a cation such as K^+ is also absorbed. The uptake of K^+ and $H_2PO_4^-$ are not directly linked. If both ions are presented to the cell at the same time the K^+ is taken up more rapidly, largely in exchange for H^+ (19), but after 10 to 15 minutes the potassium uptake slows down and the uptakes of the two ions become roughly equal.

SUMMARY

Phosphate can distribute in the cell wall space, but is not bound to an appreciable extent at the cell surface in non-metabolizing yeast. During metabolism of sugars, phosphate is actively transported into the yeast cell by a mechanism specifically involving glycolysis reactions. The movement of phosphate is in the inward direction only (no appreciable efflux), and it can proceed against a concentration gradient of 100 to 1. It is dependent on external phosphate concentrations in an asymptotic relationship, but is independent of the cellular orthophosphate concentration. The pH optimum for the phosphate uptake of 6.5 is shifted to the acid side by potassium. At certain values of pH a stimulation of 700 per cent by potassium can be observed. The nature of the effects of K^+ and H^+ are discussed.

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THE STATISTICAL DETECTION OF THRESHOLD SIGNALS IN THE RETINA

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(Received for publication, November 5, 1956)

INTRODUCTION

The statistical variations of visual responses near threshold suggest that the sensitivity of the eye, like that of many communication systems invented by man, is limited by the presence of random energy fluctuations or "noise." This noise is a result of the unpredictable motion of the individual elementary particles of physics (atoms, ions, electrons, photons) and originates either from thermal agitation or from events to which Heisenberg's principle of indeterminacy applies. If amplified sufficiently, such noise will distort the signal being communicated. (In the remainder of this paper the word noise never refers to the purely electrical noise which arises in the recording and amplifying apparatus and obscures the action potential, but only to fluctuations of physiological processes in the retina, in particular the times of occurrence of impulses.)

For the human retina, Hecht, Shlaer, and Pirenne (1942) concluded that statistical fluctuations in response to brief flashes at the absolute threshold could be accounted for entirely by random fluctuations in the number of photons absorbed by the photoreceptors at each flash, and that "biological noise" was negligible. However, the presence of large fluctuations in the duration of intervals between successive impulses of the maintained discharge from single ganglion cells in the cat's retina (Granit, 1947; Kuffler, 1952, 1953), both in darkness and for a wide range of light intensities, suggests that biological noise does play a part in limiting the sensitivity of the eye. A similar situation in the infrared receptors of the rattlesnake has been studied by Bullock and Diecke (1956).

This paper describes a statistical analysis of single ganglion cell discharges occurring in response to brief flashes of light of near-threshold intensity. The method of analysis used was suggested by a statistical theory of vision in man proposed by Tanner and Swets (1954). On the basis of this analysis the properties of the retina are described in terms of information theory. Finally, these deduced properties are shown to agree approximately with those observed in the experimental measurement of thresholds.

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Method

Single ganglion cell impulses were recorded from the eye of decerebrate cats in various stages of light and dark adaptation by methods described by Talbot and Kuffler (1952) and Kuffler (1953). Platinum wire recording electrodes were used, except that one cell (unit E) was recorded with a KCl-filled micropipette. A flashing light stimulus having an intensity controllable with a rotating wedge filter was focussed on the retina in the form of a spot of chosen diameter (usually 0.5 to 1.0 mm.) centered in the receptive field of the ganglion cell. Flashes of 5 or 10 msec. duration were delivered at a repetition frequency of one per second. Photographic records were made of the discharge during ten successive flashes at a number of different stimulus intensities near threshold, and also at zero intensity.

The ganglion cell discharge was displayed on a cathode ray oscilloscope both in the usual way as electrical potential *versus* time, and also as impulse interval duration *versus* time using the device of MacNichol and Jacobs (1955), in which the vertical scale of time is considerably magnified relative to the horizontal scale (Barlow, FitzHugh, and Kuffler, 1956 *b*). The impulses were also audible through a loudspeaker. With the flash intensity set at a certain value, the experimenter looked at and listened to the discharge, to determine whether each flash was followed by a detectable response in the form of a momentary change in frequency or in the distribution of intervals between impulses. The experimenter chose as the threshold the least intensity at which he could detect a response to roughly half the flashes. It was found necessary to let at least five successive flashes occur before making up one's mind as to whether a given intensity was threshold. Although this method required some practice, the threshold values chosen by different observers agreed to within about 0.2 $\log_{10}$ unit.

Because the response was graded rather than all-or-nothing, the thresholds measured correspond to an arbitrary reference level of excitation, convenient for testing the relative light sensitivity of the photoreceptors associated with a ganglion cell. The above method was adopted as being both as consistent and as sensitive as possible.

RESULTS

In this section both the experimental data and their statistical analysis will be presented. The purpose and significance of the latter will be taken up more fully in the Discussion section.

Data from five ganglion cells in different cats were analyzed statistically. The results from different units differed in detail, but for the purpose of illustration a complete analysis of one representative experiment will be given first. The experimental conditions required for a satisfactory statistical analysis will then be described and the differences between the different experiments mentioned. Finally the results of all the experiments will be summarized in Table I.

Sample Analysis

The data to be considered first were obtained from a single ganglion cell in a dark-adapted eye kept in total darkness except for 5 msec. testing flashes

of low intensity. The maintained discharge of this cell in the absence of light stimulation was typical; the distribution of impulse intervals fitted a gamma distribution (Pearson's type III), and the first serial correlation coefficient between successive intervals was −0.19 (Barlow *et al.*, 1956 *a*).

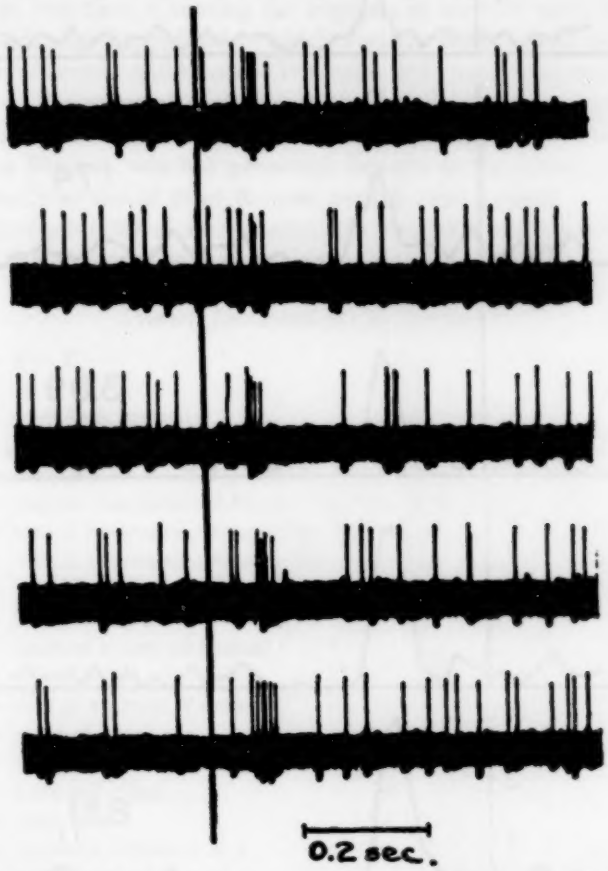

FIG. 1. Photographs of the impulses recorded from an on-center ganglion cell in response to five successive flashes given at a rate of one per second. Flash duration 5 msec.; flashes occur at long vertical line. Stimulus intensity 1.74 times threshold. The abscissa is time. Retouched photographs.

Fig. 1 shows the discharge from this cell following five successive 5 msec. flashes of intensity 1.74 times threshold, at a frequency of one per second. For the purpose of analysis, the period extending from 100 msec. before the beginning of each flash to 400 msec. after it was divided into 10 msec. periods, and for every stimulus, the impulses occurring in each of these periods counted. Then for each 10 msec. period the number of impulses was averaged over

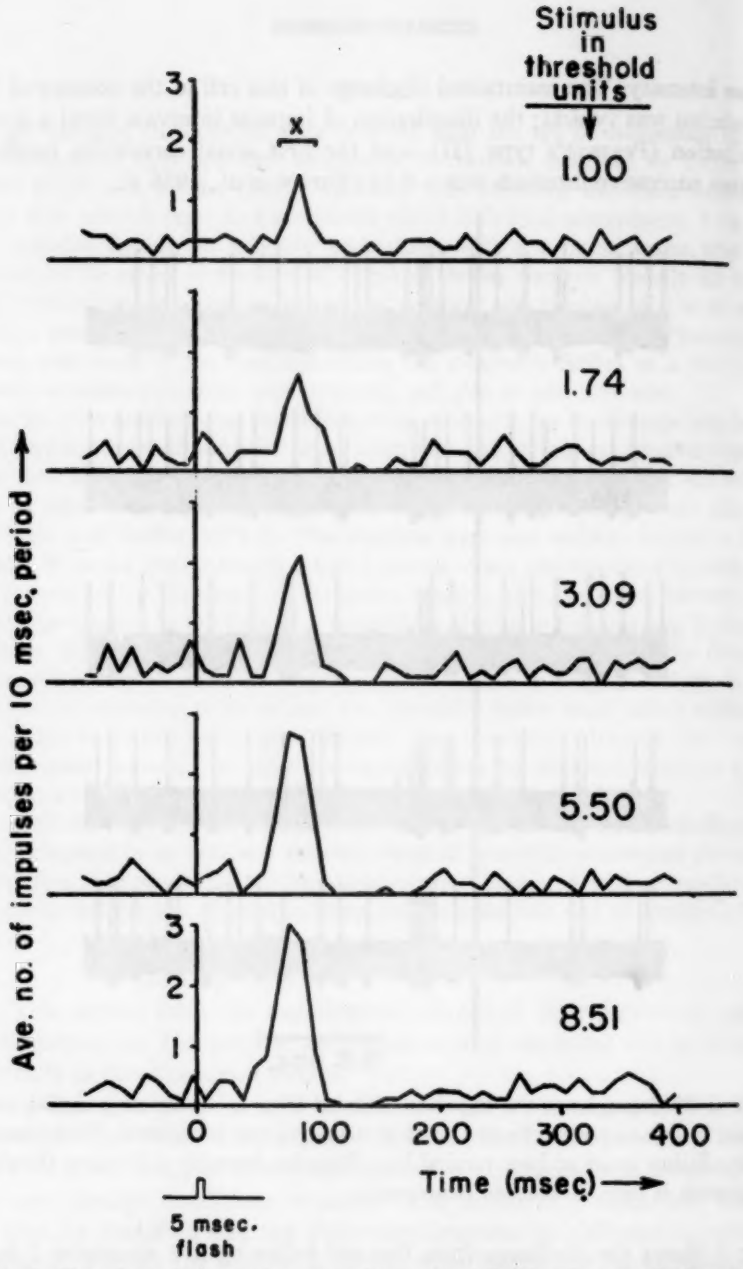

FIG. 2. Transient changes of average frequency following 5 msec. flashes, plotted from data such as those of Fig. 1. On the vertical axis is plotted the mean number of impulses in each 10 msec. period obtained by averaging over ten successive flashes. The relative stimulus intensity for each curve is as shown, with threshold taken as 1.00. The number of impulses (x) occurring in each discharge during the critical period marked at the top of the figure was used as an index of the response.

ten successive flashes. Fig. 2 shows these averages plotted against time for five different flash intensities. The intensities are expressed as multiples of the threshold intensity, estimated by the experimenter (see Methods). These curves show average impulse frequency as a function of time, with the flash occurring at zero time. Counting the impulses in each 10 msec. period is of course an arbitrary simplification which neglects variations in impulse time of the order of several milliseconds. The value of 10 msec. was, however, small compared to the range of variation of the intervals of the maintained discharge (5 to 120 msec., mean 33.5 msec., see also Fig. 1); moreover a shorter period than 10 msec. was not necessary, because in the subsequent analysis the impulses in several of these 10 msec. periods were lumped.

The following is a list of mathematical symbols used in this paper; they will be defined in more detail as used.

Symbols for Ganglion Cell Discharge

d = $\bar{x} - \bar{x}^0$
d_N = $\bar{x}_N - \bar{x}_N^0$
I = fixed flash intensity
I_θ = threshold intensity for N cells
k, K = constants
p = slope of line obtained by plotting log d against log I
R_1 = rate of information transfer for randomized flash intensities
R_n = rate of information transfer for randomized blocks of n identical flashes
w = variable flash intensity, equal to either zero or I
x = index of response
$\bar{x}$ = mean of x over 10 flashes
$\bar{x}^0$ = $\bar{x}$ for $I = 0$
x_N = sum of x's from N cells
$\bar{x}_N$ = mean of x
$\bar{x}_N^0$ = $\bar{x}$ for $I = 0$
x' = a control index
$\bar{x}'$ = mean of x'
σ = standard deviation of x
σ_N = standard deviation of x_N

Symbols for Model of Tanner and Swets

C = minimum value of X for a positive response
D = $\bar{X} - \bar{X}^0$
$f_0(X)$ = statistical distribution of X for zero intensity
$f_I(X)$ = statistical distribution of X for intensity I
P = probability of seeing flash
X = index of neural activity, corresponding to x
$\bar{X}$ = mean of X
$\bar{X}^0$ = $\bar{X}$ for $I = 0$

Fig. 2 shows that following a flash of threshold intensity there occurred, on the average, first a latent period lasting 70 msec. and then a burst of impulses lasting 30 msec. Since the duration and maximum frequency of the burst were the most obvious indices of the response, the index first chosen for analyzing the data of Fig. 2 was the number of impulses (x) occurring in the "critical period" from 70 to 100 msec. after the beginning of each flash. For the brighter flashes, the beginning of the burst occurred sooner (shorter latency), and there was also a pronounced decrease of impulse frequency ("pause") following the burst, between 100 and 200 msec. after the flash. Therefore for flashes much above threshold, the index x neglects these parts of the response. Even at threshold it neglects the detailed course of rise and fall of average frequency within the critical period. However, in the Discussion it will be shown that the use of x provides at least as efficient a way of analyzing the response as the experimental method described above.

The histograms in Fig. 3 represent the statistical distribution of x for 10 successive records at each of six different stimulus intensities (I) from zero to 8.5 times threshold. As a control at zero intensity, x was taken as the number of impulses within ten 30 msec. periods spaced 1 sec. apart in a record of the maintained discharge. The vertical line through each histogram indicates the mean, $\bar{x}$.

The index x will be compared below with the index X of Tanner and Swets (see Discussion), which has a standard deviation which is constant for all values of I. The values of the standard deviation σ of x corresponding to different values of I did not vary significantly from each other and showed no significant trend with increasing I. Details of the statistical tests used to establish this will be given in the section "Comparison of experiments."

Let d be defined as the difference $(\bar{x} - \bar{x}_0)$, in which $\bar{x}_0$ is the mean value of x for zero stimulus (Fig. 3). In Fig. 4, d is plotted against I with logarithmic scales on both axes (filled circles). A straight line with a slope of 0.73 fits the first four of the points, but not the last one. The slope of such a line will be referred to hereafter simply as the "slope," for brevity. At the highest stimulus intensity the system may be saturating somewhere; perhaps the cell body is firing near its maximum frequency.

Sampling errors of d were not estimated directly, since the slope varied rather widely with the choice of controls and of the critical period. Two types of distributions of x were used as zero-intensity controls; they will be called "control I" and "control II." Control I is that described above, in which a distribution of x was obtained from the maintained discharge; the mean value $\bar{x}_0$ taken from this distribution was used to calculate d for all values of I. The points shown as open circles in Fig. 4 were obtained by using control II, in which a control index x' was taken equal to the number of impulses within the period from -30 msec. to 0 msec. This control period is just 100 msec.

earlier than the critical period used to find x, and has the same duration, but since it ends just before the flash, x' can be used as a control index for com-

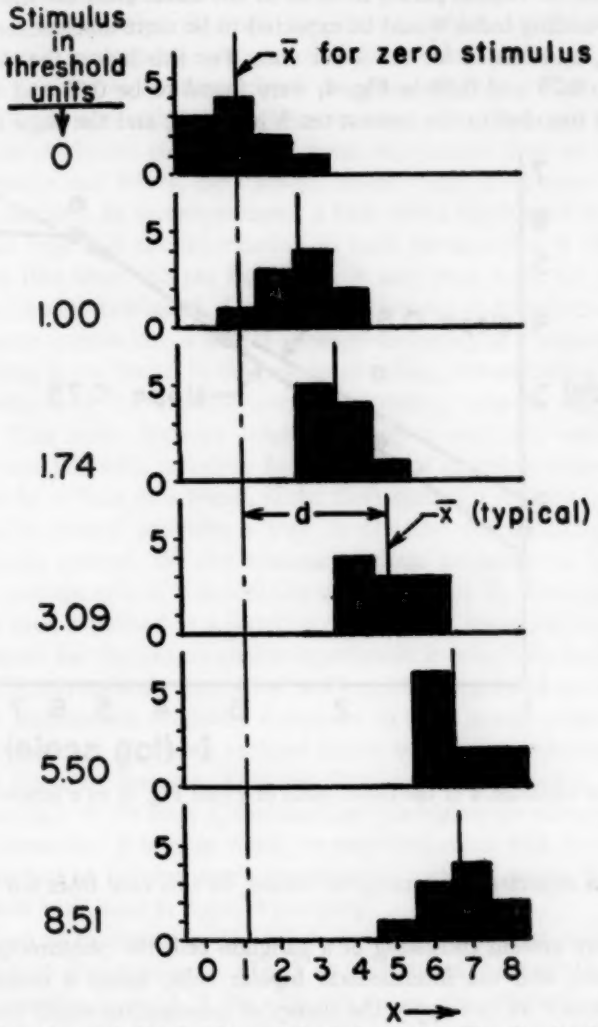

FIG. 3. Experimental distributions of the index x (see Fig. 2), obtained from records like those of Fig. 1, for six stimulus intensities.

parison with x. The mean value $\bar{x}'$ of x' was calculated; d was then taken as $(\bar{x} - \bar{x}')$ and plotted in Fig. 4 as open circles. The line drawn through these points (broken line) has the slope 0.56.

As already mentioned, the choice of the critical period from 70 to 100 msec.

after the beginning of the flash is arbitrary and was intended to reflect most efficiently the changes in response just at threshold. For the purpose of comparison, a second critical period from 40 to 100 msec. after the flash was used. The corresponding index would be expected to be more efficient for the higher values of I, and less so for the lower ones. For this index, the slopes, corresponding to 0.73 and 0.56 in Fig. 4, were found to be 0.98 and 0.64. These figures were rounded to the nearest tenth of a unit, and the slope for each ex-

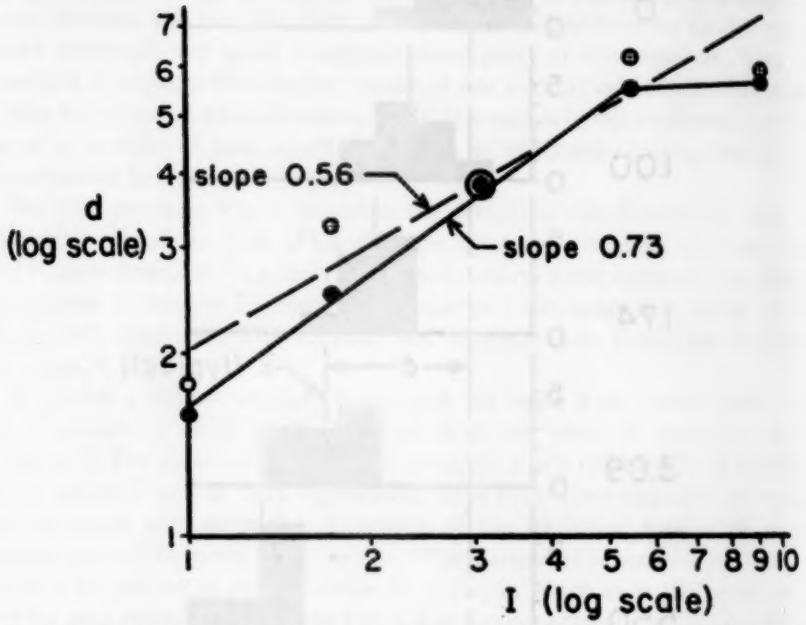

FIG. 4. The variation d of the mean value of x (see Fig. 3) as a function of stimulus intensity.

periment was expressed as a range of values, in this case from 0.6 to 1.0 (see Table I).

The sensory system consisting of a ganglion cell, the photoreceptors of its receptive field, and the intermediate bipolar cells, forms a communication channel to which we can apply the theory of information which has been developed by Shannon and others working in communication engineering (Shannon and Weaver, 1949; Goldman, 1953; Woodward, 1953). In this theory "information" is defined numerically and its unit of measurement is the "bit"—that amount of information contained in a message which specifies one of two alternatives, both of which were equally probable before the message was received (equal *a priori* probabilities). This concept of information has also been extended to more complicated cases in which the number of alterna-

tives is greater than two, their *a priori* probabilities are not equal, or the message is made up of many successive independent choices instead of just one. Information theory is useful in describing and comparing the properties of "noisy" communication channels; *i.e.*, devices which distort the messages they transmit in an unpredictable, statistical manner. In the present experiments, the message to be transmitted concerns whether a flash or a blank was presented to the retina at a given moment. Our data will be analyzed assuming the statistical conditions of visual experiment done on human subjects by Tanner and Swets, the "yes-no choice" (described more fully in the Discussion section). In this experiment, a flash and a blank have equal *a priori* probabilities (½) and the information in each presentation is therefore one bit per flash (the term "bit per flash" will be used even when the "flash" may possibly be of zero intensity). The stimulus message is transformed or coded by the sensory system into a neural message consisting of a sequence of nerve impulses going to the brain. In the process of coding, the statistical fluctuations in the durations of the impulse intervals represent noise in engineering terminology. This noise destroys information by introducing ambiguity; one cannot always tell with certainty by analyzing a neural message whether it was caused by a flash or a blank, if the flash intensity I is too low.

Information theory provides a way to describe the statistical properties of this sensory system. We can summarize these properties in a diagram in which the average rate of transmission of information R_1 through the system in bits per flash is plotted as a function of I (Fig. 5, upper curve). This curve was calculated for the yes-no choice experiment in which flashes of intensity zero and I are randomly presented and equally frequent, and the average amount of information originally contained in each presentation is therefore 1 bit. The values of R_1 shown as filled circles were computed from the distributions of Fig. 3 by formula (1) of the Mathematical appendix. R_1 increases with increasing I to the limit 1, showing that the higher the stimulus intensity, the less information is lost, as would be expected, since with increasing I the bursts and pauses become more accentuated and are easier to distinguish from random variations in impulse grouping.

The values of R_1 shown by the filled circles in Fig. 5 were calculated using the uppermost distribution in Fig. 3 as a "control," corresponding to zero stimulus intensity (control I). As with the slopes, the reliability of the values of R_1 was also checked by recalculating them using the zero-intensity control distributions provided by control II. These values are shown as open circles in Fig. 5. Values of R_1 were also calculated for the critical period 40 to 100 msec. after the flash, with both types of controls, and these are shown as filled and open squares in Fig. 5. In Fig. 5 a smooth curve drawn through the points represents average information transfer in the yes-no choice experiment as a function of flash intensity I. (It does not necessarily follow that the upper-

most value of R_1 at any I corresponds to the most efficient method of analysis; sampling errors can make R_1 either too large or too small.) The value of information corresponding to $I = 1$, or threshold intensity as estimated ex-

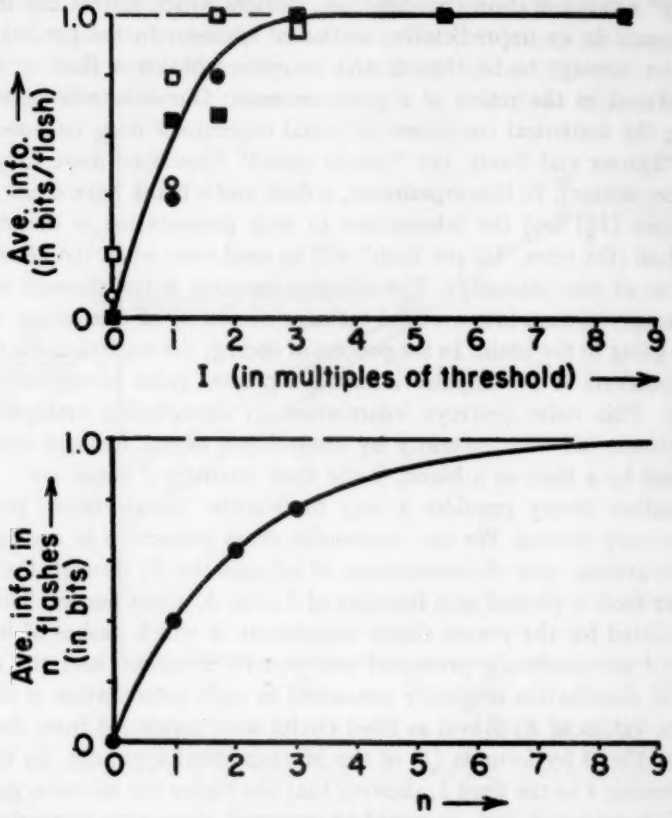

FIG. 5. Upper curve, the average information in a single flash with intensity known to be either zero or I with equal *a priori* probabilities, plotted as a function of I. Values of information computed from distributions of Fig. 3. Symbols are explained in the text. Lower curve, the average information in a sequence of n flashes known to be of the same intensity, (either zero or I, equal *a priori* probabilities), plotted as a function of n.

perimentally, is 0.4 to 0.8 bit/flash. That is, some information was being lost at this stimulus intensity, due to noise. The experimenter was, however, able to make up for this loss of information by examining the discharges for several flashes in succession; *i.e.*, by introducing redundancy into the message being transmitted. This will be made clearer by the following calculations.

Consider the case in which n successive flashes or blanks are presented in a

block. The subject knows that all the stimuli in a given block are of the *same* intensity w, but does not know whether w is zero or a fixed value I. These two values of w have equal *a priori* probability ($\frac{1}{2}$), and therefore one bit of information is contained in this block. Let R_n indicate the average amount of information in the n nerve messages resulting from such a block of flashes, for the yes-no choice experiment. R_n is expressed in bits/block. The lower diagram of Fig. 5 shows R_n as calculated from formula (5) of the Appendix for $I = 1$ (threshold intensity) and for $n = 1, 2, 3$, using the histograms of Fig. 3. (For $n = 1$, R_n is R_1, the rate for one flash considered earlier.) Values of R_n for values of n greater than 3 were not calculated because the labor of computation increases rapidly with n, but the curve of R_n *versus* n was extrapolated with an exponential curve which closely fitted to the first three points and approached the value of 1 as n approached infinity.

Calculations of R_n were not done for all the four combinations of control distributions and critical periods that were used to get the points of Fig. 5 (upper), but if this had been done, curves of similar form would have resulted. Since the method of calculation that was used gave the lowest of the values of R_1 for $I = 1$ in Fig. 5 (upper, filled circles), curves of R_n calculated in the other three ways would all lie above that shown in Fig. 5 (lower).

The increase of information with the number of identical flashes or blanks in a block agrees with the finding of Blackwell (1953) that visual thresholds in man are lower when the stimuli are presented in blocks of twenty flashes of the same intensity, than when the intensity is varied from flash to flash.

Comparison of Experiments

The five experiments will be denoted by the letters A to E. Experiment A has been analyzed in detail above.

Because all the experiments analyzed differed somewhat, it seems advisable to mention the conditions considered necessary for statistical analysis, and how the results were affected when these conditions were not completely satisfied. This occurred either when the properties of the unit were not constant during the experiment, or when the unit was lost before all the necessary data had been gathered. The conditions are as follows:—

1. The ganglion cell must have a constant frequency of maintained discharge. The frequency was monitored during the later experiments by periodically counting the impulses in a 10 second period with an electronic counter, as described by Barlow *et al.* (1956 *a*). No units were used which showed unusually large changes of maintained frequency. Moreover, as a control, two or three photographic records of the discharge, without stimulus and lasting for 10 sec., were made at the beginning, the end, and sometimes in the middle of the experiment. For unit A only one such control run was obtained, but its frequency remained quite constant in the other records, except for the response during 250 msec. following each flash (Fig. 2).

2. The retina must be in a constant state of adaptation, with a constant threshold. This was tested by making two or three records (at beginning, end, and sometimes

the middle of the experiment), using the value of the stimulus initially chosen as the threshold. Records at different stimulus intensities were made about 1 minute apart, to allow any adaptation to disappear. Any change in threshold would affect the values of $\bar{x}$ and therefore also of the slope. Again, unit A had only one such record, but had typical values for the slope. The others showed a nearly constant response to the original threshold stimulus, except for unit C. For this unit, each value of d was corrected by the amount of drift of the threshold value of $\bar{x}$, which was assumed to be changing linearly throughout the experiment. This correction changed the slope only from 0.82 to 0.75, and was unimportant compared to the other inaccuracies involved.

3. The response to one flash must be over before the next flash occurs, for the data to be applicable to the yes-no choice experiment, in which each response is used by the observer to infer the intensity of the single preceding flash only. The stimulation rate of one flash per second was usually slow enough to permit the original maintained frequency to be resumed after every flash. However, errors from this source were checked by using two sets of control distributions for x, as described earlier (controls I and II). Only in experiment D did requirement (3) fail, and only for the highest stimulus intensities, and the values of slope and information were not changed appreciably.

4. The temporal pattern of the frequency changes must be simple enough so that merely counting the impulses within a certain critical period will provide an efficient index of the response. This requirement, of course, only reflects the inadequacy of this method of analysis for the more complicated responses. Unit B had a response which consisted of a series of alternate bursts and pauses, the later ones appearing only in response to the brighter flashes (Fig. 6). Unit B was in a light-adapted retina; a similar response was noticed for one other light-adapted unit (not analyzed), but for no dark-adapted ones. These complex responses may have been the result of an interplay between mutually antagonistic sets of pathways from different parts of the receptive field (Barlow *et al.*, 1956 *b*). A systematic investigation of the effects on the frequency pattern of response of changing both the state of adaptation and the area of the stimulating spot might be informative. Unit B did not have a definite value of the slope, since a straight line could not be fitted to the points in the diagram of log d plotted against log I. This curve was concave downward with a slope varying from 0.3 to 1.6, but these limits are only slightly wider than those found for the other four units (see Table I), so that even this experiment is not in serious disagreement with the others.

Tanner and Swets assumed that the distribution of their index x was normal, with a standard deviation independent of I (see Discussion). The experimental distributions of x are not even approximately normal, since x is a discrete variable which varies by increments that are large compared to σ. However, the hypothesis that σ is constant was tested by Bartlett's test for uniformity of variance (Bartlett, 1937; Walker and Lev, 1953). This test is not strictly applicable to σ, since x is discrete, but from the discussion on rounding off of variables given in Eisenhart, Hastay, and Wallis (1947), this test is probably satisfactory, except when some of the values of σ are zero. Since approximating a continuous variable by a discrete one tends to increase the spread of sampled values of variance, this test will still be valid whenever

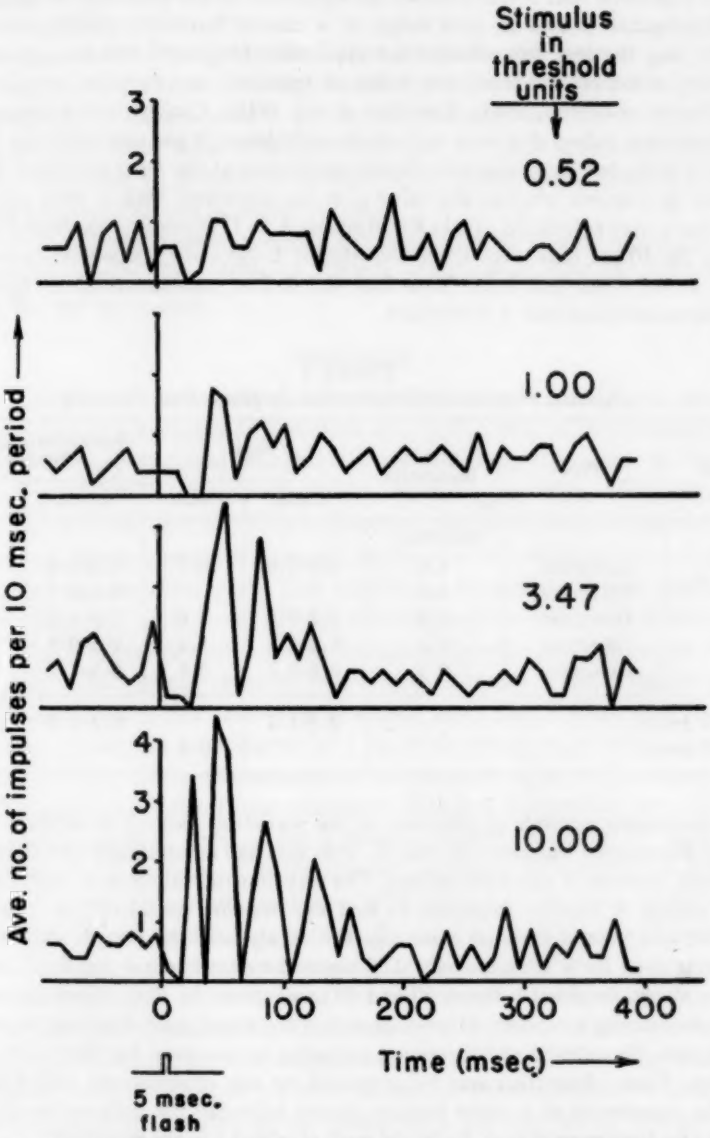

FIG. 6. Average frequency changes following a flash, plotted as in Fig. 2 for a light-adapted off-center unit. The response to the higher intensities consists of a sequence of oscillations of impulse frequency.

its result indicates non-significance. Bartlett's test, when applied to Experiments A, B, and C showed that σ was constant to within the 10 per cent level of significance. In Experiments D and E, zero values of σ caused Bartlett's statistic to become infinite, and the test was assumed not applicable. Cochran's test for uniformity of variance, based on the maximum value of variance, was therefore applied to all experiments (Cochran, 1941; Eisenhart *et al.*, 1947). Cochran's test showed that the maximum values of σ were not significantly large (5 per cent level) for Experiments A to D, but Experiment E showed significance at the 1 per cent level. Finally, in order to discover whether the value of σ was correlated with I, rank correlation coefficients were calculated. Again Experiments A to D showed no significant correlation at the 10 per cent level, while Experiment E did show a significant correlation (0.94) at the 3 per cent level. Thus four out of five experiments are in agreement with the assumption that σ is constant.

TABLE I

Statistical Properties of Ganglion Cell Responses Near Threshold

Unit	Type	Retinal illumination	Slope		Information at threshold (bits/flash)	
			Limits	Mean	Limits	Mean
		foot-candles				
A	On-center	0.0	0.6–1.0	0.7	0.4–0.8	0.6
B	Off-center	4.0			0.6–0.7	0.7
C	On-center	0.0	0.5–0.8	0.6	0.9–1.0	1.0
D	Off-center	0.3	1.1–1.5	1.2	0.5–0.8	0.6
E	On-center	0.3	0.6–0.7	0.6	0.5–1.0	0.8
Over-all limits			0.5–1.5		0.4–1.0	
Over-all mean				0.8		0.7

An interesting example of efficiency of the statistical method of analysis in preserving information was given by unit E. This unit had an unusually low maintained frequency, between 3 and 5 per second. The experimental criterion of response used, was a change in impulse *frequency*, so that the response considered as "threshold" consisted of a *pair* of impulses close together. Analysis of the records showed, however, that even for a stimulus only 0.55 times the experimental threshold, a *single* impulse always occurred between 20 and 40 msec. after the flash. Since the number of impulses during a random 20 msec. period of the maintained discharge was nearly always zero, the calculated information was close to one even for this subthreshold intensity. Thus information was being wasted by the experimenter, and if he had used the appearance of a single impulse closely following the flash as his criterion, instead of a frequency change, he would have obtained a lower threshold.

Summary of Results

In Table I, columns 4 and 5 give the limits of variation and mean values obtained by plotting log d against log I for four experiments (Experiment B

did not give a straight line—see above). In the Discussion, these figures will be compared with the corresponding values found for human vision by Tanner and Swets (1954).

In columns 6 and 7 are given the values for limits and mean of the rate R_1 of information transfer at the experimentally estimated threshold intensity (see Methods). In all but unit E, which was discussed as a special case in the last section, the mean value of R_1 was less than 1, which agrees with the fact that the responses to a number of successive flashes were observed before deciding on the threshold intensity setting.

The data in Table I were obtained by using the two control methods I and II, described above. Two different critical periods were used for units A and E, and one for the others.

DISCUSSION

The problem of the analysis of a nerve fiber message by the brain is similar to the engineering problem of detection of a signal in a noisy communication channel. A theory of signal detectability in such channels, based on the principles of statistical inference (Peterson, Birdsall, and Fox, 1954) has been used by Tanner and Swets (1954) to interpret experiments on human subjects. Tanner and Swets postulate a neural mechanism in which statistical fluctuations play an essential part. The statistical properties of this mechanism can be compared with those of the retinal ganglion cell studied here. The simplest type of experiment done by Tanner and Swets was the yes-no choice, in which at each trial the observer must report whether or not a flash occurred at a known instant. For this type of experiment, Fig. 7 illustrates their assumptions, which are as follows:—(1) At each stimulus, a certain quantity of neural activity (X, in the notation of the present paper) is the signal which the brain analyzes to determine whether a flash has occurred or not. (2) This signal contains noise; that is, for a given stimulus intensity, X varies randomly from flash to flash. X is distributed according to a normal probability curve which has a standard deviation independent of the flash intensity and taken equal to one, and a mean value $\bar{X}$ which increases with intensity. (3) Whenever X exceeds a certain threshold or criterion value C the subject reports a flash seen. (4) The criterion C varies according to the observer's knowledge of the *a priori* probability of occurrence of a flash, and also according to his degree of motivation—the subject was paid or fined according to the correctness of his reports—, and his brain adjusts C so as to maximize his average net profit. In Fig. 7, $f_0(X)$ is the probability distribution of X following a "blank" (flash of zero intensity; curve labelled "noise only"), and $f_I(X)$ is the distribution of X following a flash of intensity I ("signal plus noise"). Both curves are assumed to be normal probability curves of standard deviation (unity) but their mean values are separated by a distance D which is an in-

creasing function of I. Once the criterion C is chosen by the brain, a flash is reported seen whenever X exceeds C. For the yes-no choice experiment, the intensity of the flash and the relative frequency of flashes and blanks were controlled by the experimenter. According to their hypothesis, X can exceed C by chance when the flash is either present or absent, with probabilities which are equal to the areas under the corresponding distribution curves to

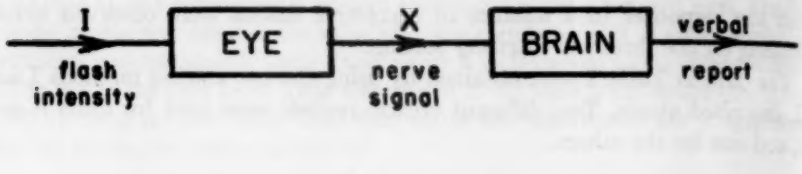

FIG. 7. Diagram illustrating the stochastic model of the visual apparatus near threshold suggested by Tanner and Swets (1954). The distribution of X is a normal probability distribution having a constant standard deviation and a mean which increases with increasing stimulus intensity I. $D(I)$ corresponds to d of Figs. 3 and 4. Whenever the value of x resulting from a flash exceeds the criterion value C, the brain reports a flash seen. The singly and doubly cross-hatched areas represent the probabilities of this happening for flash intensities of I and zero respectively. Modified from Tanner and Swets (1954).

the right of C (cross-hatched in Fig. 7) This probability is greater when a flash is present than when it is absent. If C is set very low, many blanks will be reported as flashes, while if C is high, many flashes will be reported as blanks.

By varying the conditions (mentioned above) which determine C and by analyzing the resulting data according to their theory, Tanner and Swets obtained D indirectly. When they plotted log D versus log I, they obtained straight lines with slopes of from 2.1 to 3.1, which is consistently higher than the values given in Table I (0.5 to 1.5). This difference in slopes does not

necessarily indicate a conflict between the present results and those of Tanner and Swets, since the slope might be different if x were defined in a different way. In general, one would expect that the more efficient x was in preserving information, the greater the slope would be, since then the mean value $\bar{x}$ of x would increase more rapidly with I, and since the rate of information transfer increases according to the decrease in overlap of the distributions to be distinguished. A second possible reason for the difference in slopes will be mentioned below, when the messages in many optic nerve fibers are considered.

It should be mentioned here that an analysis of the present data could have been done using the method of inference of Tanner and Swets, based on a criterion test, but the point of view of information theory was chosen as not requiring the choice of a particular test.

A sample of ganglion cell discharge can be completely described by listing the times of occurrence of each impulse in it. Because of random variation in impulse intervals, much of this data represents visually useless information, or noise, and the first step in the analysis of such data was to try to choose an index of measure of neural activity x to correspond to the index X of Tanner and Swets. This index should be both reasonably simple to calculate and efficient in the sense that little significant information is lost in passing from the original message to the quantity x. This step may be roughly compared with the practice in electrical engineering of passing a signal through a filter to emphasize those band widths in which the signal-to-noise ratio is greatest. Some analogous process of selective analysis presumably occurs in the brain as it analyzes the optic nerve messages.

In a previous paper (Barlow *et al.*, 1956 *a*) on the maintained discharge, the data analyzed consisted simply of the values of the successive intervals listed in order. For the present paper, however, the times of occurrence of the impulses were listed with reference to time after the flash, since the characteristic features of the response seemed to be more closely related to time than to ordinal number of the impulses. As described in the Results section, the index x was taken to be the number of impulses occurring during a certain critical period of time following the flash, and the amount of information per flash was calculated from the frequency distributions of x, by the formulas given in the Appendix.

When the *a priori* probabilities of a flash or a blank (*i.e.* flash intensities of zero and I) are each one-half, the average amount of information in each presentation is one bit. The average amount of information transmitted to the ganglion cell in each flash (R_1) is one bit/flash only when the intensity of the flash is so high that the response in the ganglion cell is never confused with the chance fluctuations in impulse grouping which can result from biological or quantal noise. For smaller intensities, near "threshold," there are certain values of x which can result from either a flash or a blank, and the correspond-

ing rate of transmission of information is less than one bit/flash, since some of the information in the original signal is lost through ambiguities introduced by noise. At the intensity which was judged by the experimenter to be threshold for ganglion cell A, R_1 was 0.4 to 0.8 bit/flash (Fig. 5, upper). The average information (R_6) in six successive flashes which were known to be identical, and of intensity either zero or threshold, was estimated from Fig. 5 (lower) as 0.95 bit or higher, which may be considered as near certainty. In this way some of the information lost because of noise has been restored by the redundancy in the repeated flash. Thus the statistical method of analysis is at least as sensitive as the subjective method of the experimenters, who did not make up their minds on the threshold setting until after considering five to ten flashes at one intensity.

Even though there is agreement between two quite different methods of analyzing optic nerve messages, the experimental (see Methods) and the statistical (using a numerical index of neural activity), this does not prove that either of them preserves as much of the information in the message as does a normal cat's brain. Both the experimental and statistical methods are limited in efficiency, although for different reasons. It is of course hard to compare the relative efficiencies of the cat's brain, as it analyzes a message in an optic nerve fiber entering its synapses, and of an experimenter's brain, which receives the same message, recoded, through his optic and auditory nerves. The experimenter is concentrating all his attention on the message in one fiber, while the cat is analyzing those in many fibers. The experimenter is using a highly unnatural method, aided by his experimental apparatus, while the cat is performing a task to which evolution has specifically adapted him. The mathematical method of calculating information is based on a knowledge of the statistical distributions of certain variables in the message, but this knowledge is limited by the sizes of the samples obtained from the data. The information transfer might be calculated very accurately if one knew the distribution of each impulse interval following a flash. This distribution function would not be constant in time during the response, but would contain either time or ordinal number of impulse following a flash as a parameter. The amount of data and computation required to get these distributions with sufficient accuracy would be very great. Such a method would catch more of the information in the message, but much of this would certainly be useless or noise information rather than useful stimulus information.

It is assumed here that the experimenter's actual knowledge that the flash was of intensity greater than zero did not influence his setting of the threshold. A more accurate method for measuring threshold could be used by two experimenters, if one of them were to choose flash intensities at random, either zero or I, and the other guessed the value of each intensity by observing the discharge during a block of five to ten identical flashes or blanks. To insure

statistical significance, however, this process would have to be repeated so many times at each intensity as to be very time-consuming.

In addition to suggesting a method of analyzing nerve impulse data, the hypothesis of Tanner and Swets provides a link between such data and the results of experiments on human vision. These results are often expressed as frequency-of-seeing curves, with the relative frequency of seeing a flash plotted against flash intensity. If a value of the criterion C is chosen, then for each intensity I the probability of obtaining a value of X greater than C is found directly from the theoretical distribution of X. Let P be the frequency of seeing a flash of that intensity. Then P increases with increasing I, giving a frequency-of-seeing curve of the usual sigmoid form. Moreover, P increases with decreasing C, and if C is not larger than the maximum value of X obtained when I is equal to zero, then the frequency of "seeing" will be greater than zero even in the absence of a flash, as has been found experimentally (Blackwell, 1953).

For simplicity, the present analysis has been applied only to an extremely simple case of information transfer across the retina, the yes-no choice with equal *a priori* probabilities and only a single ganglion cell considered. The maximum rate of information transfer considered, 1 bit per second, is much less than that of which a single neuron is capable. MacKay and McCulloch (1955) estimate that under certain simple assumptions the limiting capacity of a neuron with an average frequency of 50/sec. is about 450 bits/sec. This is total information and includes useless information, in the form of noise, but the useful information, due to visual signals, is probably still an appreciable fraction of this. The cases of most interest to the physiologist, however, are much more complex, and the following generalizations are possible. (1) Unequal *a priori* probabilities of flash intensities. (2) More than two intensities to be distinguished, especially discrimination in a continuum of intensities. (3) Detection of a flash when its time of occurrence is unknown to the observer. (4) Detection of temporal patterns other than short flashes; generally, the continuous inference of intensity from the nerve message. (5) The detection of spatial patterns by analysis of messages from many ganglion cells. These generalizations would introduce many theoretical and experimental complications, some of which have been considered by Tanner and Swets (1954), Tanner and Norman (1954), and Peterson, Birdsall, and Fox (1954). Only one of them will be touched on here.

Since in normal vision the neural activity which transmits visual activity to the brain occurs in a large number of optic nerve fibers, while the present data represent messages in a single fiber, conclusions from the latter cannot be compared directly with those of Tanner and Swets. In fact, increasing the number of independent noisy channels which all carry the same stimulus message will increase the information transfer at a given intensity, or decrease

the threshold intensity, as can be shown by a rough calculation. A new index x_N is defined as the sum of the x's belonging to a number N of independently firing ganglion cells. If $d = \bar{x} - \bar{x}_0$ and σ is the standard deviation of x, if d_N and σ_N are the corresponding parameters for x_N, and if

$$d = kI^p \tag{1}$$

in which p is a positive constant (equal to the slope), then

$$\begin{aligned} d_N &= Nd = NkI^p \\ \sigma_N &= N^{\frac{1}{2}}\sigma \\ \frac{d_N}{\sigma_N} &= \frac{N^{\frac{1}{2}}kI^p}{\sigma} \end{aligned} \tag{2}$$

Assume that a certain value of average information transfer R_1 represents threshold vision and corresponds to a fixed value of d_N/σ_N. Let us keep R_1 and therefore d_N/σ_N, fixed, and consider I_θ, the threshold intensity, as a function of N:

$$I_\theta = KN^{-1/2p} \tag{3}$$

in which

$$K = \left(\frac{\sigma}{k}\cdot\frac{d_N}{\sigma_N}\right)^{1/p} = \text{a constant}$$

If the average value of $p = 0.8$ from Table I is substituted into (3), then

$$I_\theta = KN^{-0.63}$$

From this formula we can calculate for example that the threshold intensity for 100 ganglion cells would be 1.24 $\log_{10}$ units less than (or 0.058 times) that for one ganglion cell.

According to equation (2), x_N has the same value of slope as does x. Of course it may be that formula (1) does not hold over such a wide range of intensities. Perhaps as I increases, the response begins to saturate and p decreases, which might account for the fact that the value of the slope found here is lower than that found by Tanner and Swets. If their maximum value for p of 3.11 is substituted into formula (3), then the threshold value for 100 ganglion cells is only 0.32 $\log_{10}$ unit less than (or 0.48 times) that for a single cell. From these rough calculations it can be seen that the effectiveness of summation among many ganglion cells at threshold depends rather sensitively on the value of p, indicating that the value of this parameter plays an important part in determining the properties of the theoretical model of the retina.

SUMMARY

1. Photographic records of impulses from single ganglion cells in the cat's retina were made while the retina was stimulated by flashes occurring once a second. Ten flashes at each of several intensities near threshold were used.

2. For the purpose of statistical analysis, the number of impulses (x) falling within a critical period following each flash was used as an index of the response. Histograms of x were plotted and used to calculate rates of transfer of information by the ganglion cell for the case of an ideal experiment, the yes-no choice, in which flashes of intensity I and blanks are to be distinguished.

3. The information rate increased (*a*) with increasing stimulus intensity and (*b*) with the number of identical flashes or blanks presented successively in a block. The intensity chosen as threshold by the experimenter, who observed the impulses visually and aurally, corresponded to an average information rate for single flashes of 0.7 bit/flash, compared to the maximum possible rate of 1 bit/flash. A threshold intensity giving 0.4 or more bit/flash, if presented in blocks of six identical flashes, corresponded to 0.95 or more bit/block, or near certainty. Thus the calculation of information rates using the index x provides an estimate of threshold at least as sensitive as those obtained during an experiment, which were made only after observing the responses to five to ten flashes of the same intensity.

4. The index x has statistical properties similar to those of the "index of neural activity" used by Tanner and Swets (1954) in their statistical model of human vision, and represents a possible physical interpretation of their index. However, x gave values (0.5 to 1.5) of the parameter called the slope which were consistently smaller than their values (2.1 to 3.1).

I wish to thank Dr. S. W. Kuffler, in whose laboratory this work was done, for suggesting the problem considered in this paper. The experiments were done with Dr. H. B. Barlow, Dr. K. T. Brown, and Dr. T. N. Wiesel. Preliminary discussions of the problem with Dr. Barlow were very valuable.

This work was supported by a grant from the National Institutes of Health, United States Public Health Service.

MATHEMATICAL APPENDIX

Symbols Used Only in Appendix

$f_w(x)$	= conditional probability of x, given w
$H(w)$, $H(x)$	= entropies of distributions of w and x
$H(w, x)$	= entropy of joint distribution of w and x
$p(w)$	= probability of w
$q(x)$	= probability of x

The formulas used to calculate the average rate of transmission of information in the optic nerve fiber message will be derived here. In the ideal experiment (yes-no choice) discussed in this paper, a flash intensity (I) is chosen, and a sequence of flashes and blanks is presented to the retina. w denotes the flash intensity for a particular presentation; for a flash, $w = I$, and for a blank, $w = 0$. The probabilities of a flash or a blank at a given presentation are both equal to $\frac{1}{2}$. The variable x denotes the index of neural activity chosen

for analysis of the data, namely the number of impulses occurring within a certain critical period of time following the flash. The rates of information to be calculated are therefore applicable to an observer who receives only the value of x, but not the entire message.

Let $f_w(x)$ be the conditional probability that the index will have the value x, given that the flash intensity was w. Then

$$\sum_x f_w(x) = 1,$$

in which x has the range of values 0, 1, 2, 3,. . . . . The joint probability of occurrence of a given pair of values of w and x at a given presentation is $\frac{1}{2}f_w(x)$. The probabilities $p(w)$ and $q(x)$ of w and x are, respectively:

$$p(w) = \sum_x \tfrac{1}{2}f_w(x) = \tfrac{1}{2}$$

$$q(x) = \sum_w \tfrac{1}{2}f_w(x) = \tfrac{1}{2}\sum_w f_w(x)$$

From these probabilities are calculated three entropies: $H(w)$, the entropy of w; $H(x)$, that of x; and $H(w,x)$, the joint entropy of w and x. (All logarithms are to the base 2.)

$$H(w) = -\sum_w p(w) \log p(w) = 1$$

$$H(x) = -\sum_x q(x) \log q(x)$$

$$H(w, x) = -\sum_w \sum_x [\tfrac{1}{2}f_w(x)] \log [\tfrac{1}{2}f_w(x)]$$

$$= 1 - \tfrac{1}{2}\sum_w \sum_x f_w(x) \log f_w(x).$$

Let R_1 (I) be the average rate of transmission of information in bits/flash for the ideal experiment; it is a function of I. Then (Shannon and Weaver, 1949):

$$R_1(I) = H(w) + H(x) - H(w, x). \tag{4}$$

The ideal experiment can be modified so that instead of one flash, n successive flashes are presented in a block, known by the observer to be all of the same intensity w, either zero or I, with equal probability.

Let $x_1, x_2, \ldots, x_n$ be the values of the index obtained from the n messages. The joint probability of $w, x_1, x_2, \ldots, x_n$ is

$$\tfrac{1}{2}f_w(x_1)f_w(x_2) \cdots f_w(x_n).$$

The probability $p(w)$ of w is $\frac{1}{2}$, and the joint probability of $x_1, x_2, \ldots,$ and x_n is

$$q(x_1, x_2, \cdots, x_n) = \tfrac{1}{2}\sum_w f_w(x_1)f_w(x_2) \cdots f_w(x_n).$$

The corresponding entropies are

$$H(w) = 1$$

$$H(x_1, \cdots, x_n) = -\sum_{x_1} \cdots \sum_{x_n} [\tfrac{1}{2} \sum_{w} f_w(x_1) \cdots f_w(x_n)] \log [\tfrac{1}{2} \sum_{w} f_w(x_1) \cdots f_w(x_n)]$$

$$= 1 - \tfrac{1}{2} \sum_{x_1} \cdots \sum_{x_n} [\sum_{w} f_w(x_1) \cdots f_w(x_n)] \cdot \log [\sum_{w} f_w(x_1) \cdots f_w(x_n)]$$

$$H(w, x_1, \cdots, x_n) = -\sum_{w} \sum_{x_1} \cdots \sum_{x_n} [\tfrac{1}{2} f_w(x_1) \cdots f_w(x_n)] \cdot \log [\tfrac{1}{2} f_w(x_1) \cdots f_w(x_n)]$$

$$= 1 - [\sum_{w} n \sum_{x} \tfrac{1}{2} f_w(x) \log f_w(x)]$$

$$= 1 + n[H(w, x) - 1].$$

$R_n(I)$, the average information rate in bits/block, is

$$R_n(I) = H(w) + H(x_1, \cdots, x_n) - H(w, x_1, \cdots, x_n) \tag{5}$$

In calculating $R_1(I)$ and $R_n(I)$ from the experimental data, $f_w(x)$ was assumed equal to the experimental distributions of x, as given for example in Fig. 3.

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VOLUME 40, No. 6 JULY 20, 1957

PUBLISHED BIMONTHLY

AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE 2, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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Entered as second class matter November 25, 1918, at the Post Office at Baltimore, Md., under the act of March 3, 1879

Made in the United States of America

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